

# Extrachromosomal Inheritance in Bacteria

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## INTRODUCTION

In view of the demonstrations that such widely diverse species as *Escherichia coli* (106), *Bacillus subtilis* (59), *Mycoplasma hominis* (19), and *Streptomyces coelicolor* (120) have but a single chromosome, it seems probable that the genome of any prokaryotic species consists essentially of a single major linkage group, the chromosome, containing all or nearly all of the cell's genes. Many types of bacteria are known to have additional facultative linkage groups separate from their chromosome, of much smaller size, which are referred to collectively as extrachromosomal elements. Although these facultatively hereditary units are structurally and functionally analogous to chromosomes, they can be conveniently characterized as extrachromosomal because of their nonessentiality and their small size. This review will be centered on bacterial extrachromosomal elements, for the most part exclusive of temperate phages, and will deal extensively with the *Enterobacteriaceae* and with *Staphylococcus aureus*. Survey of the published literature extends through March 1969.

Other recent reviews have effectively covered a considerable part of the area of extrachromosomal inheritance in bacteria. These include the now classic paper of Campbell (31) focusing on  $\lambda$  phage, more recent reviews by Reeves (212) and Bradley (23) on colicins, reports by Scaife (230) focusing on F integration and F-merogenote formation, Falkow et al. (71) on molecular biology of episomes and conjugational transfer, Meynell et al. (172) on taxonomic relationships among transmissible plasmids, Watanabe (270, 271) and Mitsuhashi (174) on R factors, and Richmond (217) on penicillinase plasmids.

The present review will emphasize the genetic analysis of extrachromosomal elements, an area not thoroughly covered by other reviewers, and it will be more analytical and critical than comprehensive.

## Definitions

Because the terminology of bacterial genetics has become rather intricate and as certain terms have been used differently by different writers, it is necessary to offer several definitions.

"Extrachromosomal" will be used operationally to refer to hereditary units that are physically separate from the chromosome of the cell.

An "extrachromosomal element" is a stably inherited component of the cell genome when physically separate from the chromosome; e.g., F is considered an extrachromosomal element even when, in an Hfr strain, it is integrated; an abortive transducing fragment is not an extrachromosomal element since it is not a stable component of the cell genome. Coliphage P1 would be an extrachromosomal element according to this definition. Coliphage lambda would not be one. The terms "extrachromosomal element" and "plasmid" (151) will be used as synonyms. "Episome" will refer to a class of plasmids that can exist in a state of integration into the chromosome of their host cell as well as in an autonomous state (135). Note that the term episome refers in essence only to those activities or functions of a plasmid that are specifically related to chromosomal integration. Thus, the term is probably more useful as an adjective than as a noun (e.g., the "episomal" functions of F have not been observed to occur in *Proteus* strains).

Some writers (98, 132) use the term plasmid to refer to extrachromosomal elements that are *incapable* of being integrated into the host chromosome. This usage is undesirable because of the operational problem of proving that a given element is incapable of integration and because it gives rise to such semantic whimsies as "one man's episome is another man's plasmid" (71). Furthermore, Lederberg, who first used the word, suggested it as a generic term for all extrachromosomal hereditary units (151).

A "transmissible plasmid" is a plasmid that carries genetic determinants for its own intercell transfer via conjugation. A "sex factor" is a transmissible plasmid that has the capacity to promote the transfer of genetic units not linked to itself, especially the chromosome (35). Because most transmissible plasmids are sex factors, this distinction may turn out to be purely theoretical.

A "quiescent plasmid" is one whose replication is specifically controlled so as to occur once per cell division cycle. A "vegetative" plasmid is one whose replication is not under any such specific control and is restricted, if at all, only by non-specific metabolic limitations such as the availability of precursors, energy, or replicating enzymes. A "lethal" vegetative plasmid is one whose unrestricted replication per se results in death of the host cell. Intermediate forms occur (*see* "Autonomy and Infectivity") in which plasmid replication appears to be partially but not stringently controlled.

Table 1 lists the abbreviations and symbols used in this review, not all of which are in general use. In referring to genetic traits, etc., I will conform in general to the nomenclature proposed by Demerec et al. (54); where this and other nomenclature is different from that in general usage, I will note the latter parenthetically.

#### Overall Genetic Organization of a Plasmid

All of the well-studied extrachromosomal elements in bacteria are independent replicons as defined by Jacob and Brenner (129), consisting of double-stranded deoxyribonucleic acid (DNA) molecules. It must be borne in mind, however, that information on chemical nature is not available for all plasmids; the unlikely possibility remains that there may be some plasmids composed of substances other than double-stranded DNA.

The basic minimum requirement for a plasmid-like existence would appear to be equipment for autonomous replication coordinated with the division cycle of the host cell and for equitable distribution of replicas to daughter cells. A reasonable hypothesis is that the structure of a plasmid includes a recognition site or sites for initiation (or repression) of replication and a site for attachment to a structural component of the cell (a maintenance site) through which plasmid replication is keyed to the cell cycle and replicas are properly distributed (130). Diffusible substances, such as enzymes, involved in replication could be supplied by the host or by the plasmid—different plasmid systems may vary in this respect.

Theoretically, functions involved in quiescent autonomous replication and distribution are the only ones essential for plasmid survival and may occupy only a small portion of the plasmid genome. The remainder would consist of determinants of a variety of functions that are nonessential for the quiescent existence of the plasmid but are either expressed in the host cell phenotype or are involved in vegetative plasmid replication.

In the staphylococcal penicillinase plasmids, it appears that all of the essential functions are confined to a particular region of the plasmid genome with no nonessential functions interspersed (192). Many authors have assumed, without direct genetic evidence, that a similar topographical separation of functions exists in R factors and other sex factors (172, 174, 270).

#### RECOGNITION AND IDENTIFICATION OF EXTRACHROMOSOMAL ELEMENTS

Because of the advanced state of genetic analysis among the *Enterobacteriaceae*, particularly *E. coli*, the recognition of extrachromosomal inheri-

TABLE 1. *Symbols and abbreviations*

Symbol	Explanation
<b>Plasmids</b>	
F	Original <i>E. coli</i> sex factor (exclusive usage)
F'	F incorporating a segment of host chromosome; an F-merogenote
F <i>lac</i>	F incorporating the <i>lac</i> region
F- <i>gal</i>	F incorporating the <i>gal</i> region
Col	Colicinogenic factor
F $\beta$ , $\Delta$ , WG-4	Other sex factors
R	Plasmid carrying resistance determinants
R(nt)	Nontransmissible R factor
R(t)	Transmissible R factor
R(i), (R, fi) <sup>a</sup>	R(t) factor with I specificity
R(f), (R, fi <sup>+</sup> )	R(t) factor with F specificity
Amp (A)	R(nt) factor carrying ampicillin resistance
Str-Sul (SSu)	R(nt) factor carrying streptomycin and sulfonamide resistance
Tet (T)	R(nt) factor carrying tetracycline resistance
Kan (K)	R(nt) factor carrying kanamycin resistance
Chl (C)	R(nt) factor carrying chloramphenicol resistance
PI	Penicillinase plasmid, <i>S. aureus</i> (Fig. 3)
PII	Penicillinase plasmid, <i>S. aureus</i> (Fig. 3)
pi-his	Cryptic plasmid incorporating the histidine region
Hly	Plasmid incorporating <i>hly</i>
Ent	Plasmid incorporating <i>ent</i>
K88	Plasmid incorporating <i>K88</i>
<b>Genetic markers</b>	
<i>acr</i>	Affecting resistance to acridines
<i>amp</i> (A)	Affecting response to ampicillin
<i>asa</i> (AsO <sub>4</sub> <sup>-</sup> )	Affecting response to arsenate
<i>asl</i> (AsO <sub>3</sub> <sup>-</sup> )	Affecting response to arsenite
<i>att</i>	Chromosomal attachment site
<i>bis</i> (Bi <sup>+++</sup> )	Affecting response to bismuth
<i>bio</i>	Biosynthesis of biotin
<i>C<sub>I</sub></i>	Affecting ability to lysogenize
<i>cad</i> (Cd <sup>++</sup> )	Affecting response to cadmium
<i>chl</i> (C, CM)	Affecting response to chloramphenicol
<i>col</i>	Production of colicin
<i>cor</i>	Affecting response to colicin
<i>drd</i>	Derepressed (referring to mating activity of sex factors)
<i>ero</i> (E, EM)	Affecting response to erythromycin
<i>eex</i>	Entry exclusion of mating
<i>fer</i>	Conjugal transmissibility
<i>his</i>	Biosynthesis of histidine
<i>ile</i>	Biosynthesis of isoleucine
<i>ilv</i>	Biosynthesis of isoleucine and valine
<i>kan</i>	Affecting kanamycin resistance
<i>lac</i>	Fermentation of lactose
<i>lea</i> (Pb <sup>++</sup> )	Affecting response to lead
<i>leu</i>	Biosynthesis of leucine
<i>mcr</i> (mc)	Maintenance, compatibility, and replication
<i>mer</i> (Hg <sup>++</sup> )	Affecting response to mercury
<i>met</i>	Biosynthesis of methionine
<i>mod</i>	Modification of (phage) DNA
<i>neo</i> (N)	Affecting response to neomycin
<i>pen</i> (P, PC)	Affecting response to penicillin
<i>pil</i>	Production of sex pili
<i>pro</i>	Biosynthesis of proline
<i>rec</i>	Affecting recombination

TABLE 1.—*Continued*

Symbol	Explanation
<i>rep</i>	Replication
<i>res</i>	Restriction of (phage) DNA
<i>seg</i>	Affecting segregation of a plasmid
<i>sil</i>	Affecting resistance to silicate
<i>spp</i>	Suppression of plaque formation
<i>str</i> (S, Sm)	Affecting response to streptomycin
<i>sul</i> (Su)	Affecting response to sulfonamide
<i>sup</i>	Suppressor
<i>tau</i>	Affecting response to phage $\tau$
<i>tet</i> (T, Tc)	Affecting response to tetracycline
<i>trp</i>	Biosynthesis of tryptophan
<i>uvr</i>	Affecting resistance to ultraviolet
<b>Phenotypic traits</b>	
ChlR	Resistance to chloramphenicol
TetR	Resistance to tetracycline
StrR	Resistance to streptomycin
SulR	Resistance to sulfonamide
Lac <sup>+</sup> , Lac <sup>-</sup>	Ability to ferment lactose, inability
Trp <sup>+</sup> , Trp <sup>-</sup>	Ability to make tryptophan, inability
Leu <sup>+</sup> , Leu <sup>-</sup>	Ability to make leucine, inability
Pro <sup>+</sup> , Pro <sup>-</sup>	Ability to make proline, inability
Hft	High-frequency transducing
HFCT	High-frequency colicin transferring
Tsr	Thermosensitive for replication
<b>Miscellaneous</b>	
SDS	Sodium dodecyl sulfate
UV	Ultraviolet
G + C	Guanine + cytosine
EMS	Ethyl methane sulfonate
RF	Replicative form
RTF	Resistance transfer factor

<sup>a</sup>Items in parentheses are synonyms in current or recent use.

tance in these organisms has become relatively straightforward. Probably for this reason, little attention has been devoted in the literature specifically to problems surrounding the identification of extrachromosomal elements in other genera, where the situation is often much less clear. Consequently, the current state of affairs is rather unsatisfactory; different investigators use different criteria with varying degrees of validity. It is hoped that a brief discussion of the criteria, as outlined in Table 2, will clarify matters somewhat.

The general problem that most often presents itself is that a bacterial strain exhibiting a particular phenotypic trait gives rise to variants that no longer exhibit that trait. What has to be decided is whether the genetic determinant of the trait in question is chromosomal, in which case the negative variants represent deletions, point mutations, or phase variations (153) involving it, or whether the determinant is linked to a plasmid of which the variants have lost all or part (270). It will be recognized that, when plasmids are involved in such situations, they must be nonessential for cell viability. The question of whether

essential plasmids exist and, if so, how they may be recognized is considered separately (see "Essential Plasmids").

#### Lack of Genetic Linkage to Chromosome

The basic criterion for the identification of an extrachromosomal element is the demonstration that some genetic determinant is unlinked to the host cell chromosome. This criterion, however, can be applied only to those few species that have well-mapped chromosomes or participate in mating, or both. In such species, extrachromosomal inheritance can usually be ruled out by the demonstration of consistent linkage to known chromosomal loci. An exception to this rule would be a converting phage with a constant integration site, but in that case, its extracellular transmission would reveal its true nature.

#### Lack of Genetic Homology with Chromosome

It is generally true that naturally occurring plasmids do not have overall genetic homology with their host chromosome, although limited regions of homology may be present. This statement is based on the failure of such elements to combine freely with the chromosome; for example, the chromosome is unable to rescue markers from ultraviolet (UV)-inactivated plasmids (7, 161, 189).

Lack of homology with the host chromosome provides plasmids with at least two genetically demonstrable properties that are not shared by chromosomal regions of comparable size. Since donor plasmid alleles are not eliminated through recombination after transfer to a plasmid-negative host, these markers often show very high linkage to one another in conjugational (273) and transductional (196) crosses; sets of markers that show this high linkage also show very high rates of coelimination.

#### Occurrence of Conjugal Transfer

Among the *Enterobacteriaceae*, sexual promiscuity is a hallmark of extrachromosomal inheritance: the known fertility determinants are all carried by plasmids; given a pair of fertile strains it is fair to assume that, until proven otherwise, at least one is carrying a transmissible plasmid.

#### Demonstration of Physical Autonomy

If a gene is plasmid-linked, plasmid-specific DNA should be demonstrable in cells carrying it and absent from cells lacking it. In favorable cases, plasmid DNA will have a buoyant density different from host chromosomal DNA (281), or it may be transferred to a host with a different DNA base composition (165). In such cases, the

TABLE 2. *Criteria for establishing plasmid linkage*

A. Lack of genetic linkage to chromosome
B. Lack of genetic homology with chromosome
C. Occurrence of conjugal transfer
D. Demonstration of physical autonomy
E. Demonstration of replicative autonomy
Ability to be transferred to a recombination-deficient organism
Isolation of replication- or segregation-defective mutants <sup>a</sup>
Radiation target size <sup>a</sup>
UV inactivation kinetics (transduction)
Incompatibility with a known plasmid
Plasmid loss and curing <sup>a</sup>

<sup>a</sup> Application does not require genetic transfer.

plasmid DNA can be identified as a "satellite" band in a centrifugal buoyant density gradient. Alternatively, in so far as plasmid genomes consist of closed circular duplex DNA (see Table 6), they are separable from bulk (chromosomal) DNA by certain intercalating dyes used in conjunction with pycnographic techniques (44, 211) or by reversible alkali denaturation (136, 226).

Occasionally plasmids are identified physically before there is any genetic reason to suspect their existence. The recent literature includes four examples of genetically silent plasmid-like DNA (see "Cryptic Plasmid DNA"); there will doubtless be many others.

#### Demonstration of Replicative Autonomy

**Ability to be established in a recombination-deficient host.** Transferred chromosome fragments of the size usually participating in bacterial genetic transfer depend for stable survival upon a functional recombination system in the recipient. Plasmids, if transferred intact, are autonomously functioning entities that do not in theory depend for their survival on host recombination systems. For example, the sex factors of coliform organisms are readily transferred to *rec*<sup>-</sup> hosts, either by conjugation (36) or by transduction (203). It is expected that other plasmids in these and other organisms will behave similarly, though none has so far been tested. (This discussion applies to conjugation and transduction; the transformable species have so far failed to yield an identifiable plasmid.)

With these qualifications in mind, I should like to suggest as a rule that, with one exception, any trait that shows similar rates of transfer to *rec*<sup>+</sup> and *rec*<sup>-</sup> recipients is plasmid-linked. The exception is that chromosome fragments carrying the *rec*<sup>+</sup> allele will also show this behavior; however, involvement of the *rec*<sup>+</sup> allele can easily be recognized. The converse of this rule, namely,

that traits incapable of being transferred to a *rec<sup>-</sup>* host are chromosomal, is likely to be somewhat less useful as a criterion, since situations can be envisioned in which traits that are plasmid-linked may nevertheless fail to be transferred to the *rec<sup>-</sup>* host. For example, in a transduction system, a plasmid might be too large to be carried by a phage, and its transduced fragments might resemble chromosome fragments in their behavior, especially if homology with a chromosomal region is sufficient to allow crossing over and integration.

**Isolation of replication- or segregation-defective mutants.** Plasmids, in functioning independently, are likely to utilize specific mechanisms for their maintenance and replication. In so far as these mechanisms are different from those involved in chromosome maintenance and replication, it will be possible to isolate mutations affecting plasmid autonomy but not affecting the overall viability of the host cell.

This hypothesis was first proposed by Jacob et al. (130), who demonstrated its validity by isolating thermosensitive mutations affecting specifically the survival of F-*lac* in a normal host strain. These mutants, some with plasmid-linked mutations and some with host-linked mutations, segregated F-*lac* negatives with increasing frequency during growth at increasing temperature, demonstrating unequivocally the hereditary autonomy of the F particle. Although similar mutants have been isolated for staphylococcal penicillinase plasmids (193), extensive effort in a number of laboratories has failed to produce any involving R factors (Y. Hirota, *personal communication*; T. Watanabe, *personal communication*). Thus, it may not be possible to isolate such mutants for all plasmids but, in those cases where it is possible, they provide strong evidence for the plasmid state.

The application of this criterion in practice, for a trait suspected of being plasmid-linked, involves the attempt to isolate mutants that lose the trait irreversibly during growth at elevated temperatures. It may be superfluous to consider the possibility of finding conditional mutations affecting the hereditary survival of a chromosomal region, but, in the absence of any pertinent experimental data, it may be prudent to avoid assuming categorically the contrary.

**Radiation target size.** Various kinds of radiation can inactivate an entire replicon and all genes linked to it. The relative frequency of inactivation events (lethal hits) is a measure of the relative size of a radiation-sensitive target. For example, a plasmid may be 1% of the size of the host cell chromosome, whereas a single chromosomal gene may be some 100-fold smaller. In an irradiated

population of bacteria receiving an average of X lethal hits per cell, if hits are Poisson-distributed to all cellular DNA, the plasmids will have received an average of X/100 lethal hits, and any chromosomal gene will have received a maximum of X/10<sup>4</sup> hits. Of the surviving cells,  $e^{-X/100}$  will have lost their plasmid, whereas of the order of  $e^{-X/10^4}$  will have sustained an inactivating hit in any given gene. If X is about 10, some 10% of survivors will be plasmid-negative, whereas mutations affecting a given chromosomal gene will be present in only about 0.1%. This example is an oversimplification, based on the assumptions that only one copy of a plasmid will be present, that mutagenic hits occur with roughly the same probability as lethal hits and that hits in a plasmid are not lethal for the host (29); it is presented to give an idea of the magnitudes involved and to illustrate the method.

**Kinetics of radiation inactivation in gene transfer experiments.** Radiation target sizes have usually been measured in gene transfer systems to circumvent the problem of multiple copies and to permit direct study of the plasmid in an unirradiated host. Relatively large single-hit targets have been demonstrated with radiophosphorus for F and colicinogenic factors in conjugational crosses (58, 149, 204, 256), with UV irradiation of phage in transductional crosses involving F and  $\lambda$  prophage (7), penicillinase plasmids, (189) and tetracycline plasmids (8, 210), and with radiophosphorus labeling of penicillinase plasmids in transductional crosses (R. Novick, *unpublished data*).

With UV-inactivation of transducing activity, not only will a chromosomal gene be much less sensitive than will a plasmid-linked gene under the same circumstances (7, 161, 189), but also transducing activity for the chromosomal gene will be stimulated by low UV doses (84). This stimulation will not be seen for plasmid transduction since it evidently involves an enhancement of recombination; transducing fragments that would ordinarily be lost as abortives are converted to stable recombinants (16).

**Incompatibility with a known plasmid.** Incompatible plasmids are unable to reside stably in a single cell (60, 195, 231). Thus, if a known plasmid is introduced into a strain carrying a possible plasmid-linked trait and the latter becomes unstable or is lost as a direct consequence of the introduction of the former, it may be concluded that the trait in question is plasmid-linked. A negative result in this test, however, is unenlightening since most naturally occurring plasmids are compatible with one another (*see* Fig. 1 and "Fertility").

**Plasmid loss and curing.** Probably all bacterial

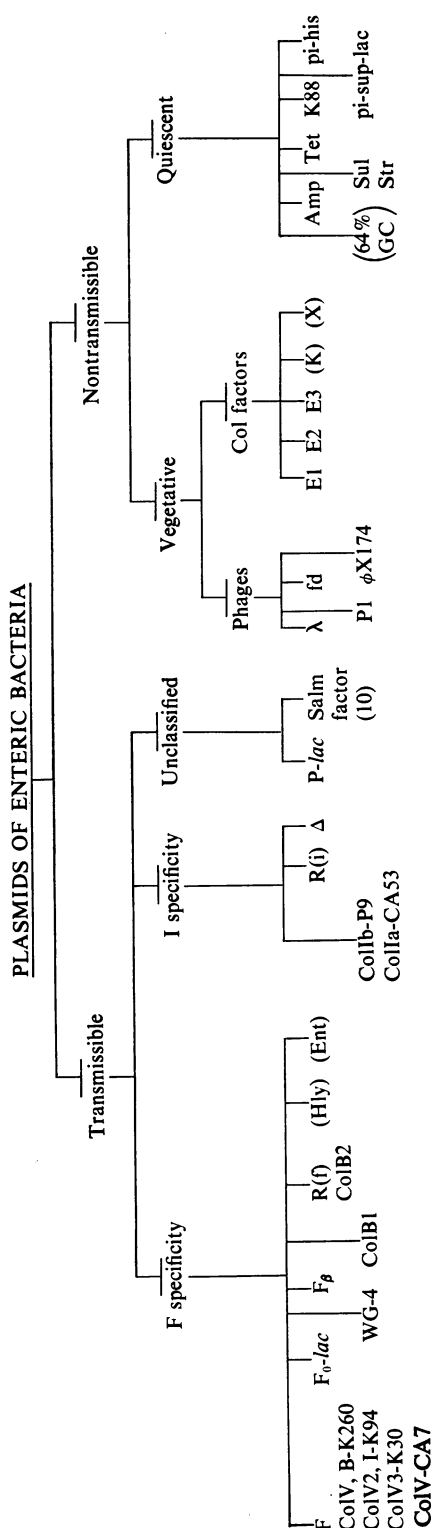


FIG. 1. Classification of plasmids harbored by enteric bacteria. This scheme is an amplification of that of Meynell *et al.* (172). See Table 4 and text for general explanation. Parentheses for Hly, Ent, and 64% G+C indicate that transmissibility is not certain; those for Col K and Col X indicate that there is reason to question their compatibility (142). The plasmids grouped as "quiescent" are only inferred to be so—direct information is not available.

strains carrying nonessential plasmids throw off plasmid-negative variants as a result of occasional errors in plasmid replication or segregation. The frequency of such variants can often be increased by certain physical or chemical agents—notably, elevated temperature (169, 262), thymine starvation (40), mutagens (280), nickel and cobalt (113), acridines (114), and other intercalating dyes (245). This effect is often referred to as "curing" of plasmids, by which is implied that the plasmid is selectively inactivated or inhibited in replication. I feel that the term should be reserved for situations in which direct selective action on the plasmid has been clearly demonstrated; I have set it in quotes in the following discussion when referring to situations in which this is not the case.

The interpretation of negative variants and the increase of their occurrence by these agents as representing plasmid loss is risky, unless one can rule out either (i) other sources of hereditary variation or (ii) selective or mutagenic effects (or both) of the various agents.

Among other sources of hereditary variation, namely, point mutations, phase variations (153), and deletions, it is in theory easy to rule out the two former since they are reversible. In practice, it is sometimes difficult to eliminate hidden selective effects which may militate against the recovery of reversions and thus make point mutants or phase variants seem irreversible. For example, it is common knowledge that pathogenic organisms sometimes lose their virulence irretrievably on subculture in the laboratory. Such irreversibility could represent either the loss of a plasmid responsible for virulence or an intrinsically reversible variation in which the laboratory environment is strongly selective in some unknown way for avirulence.

To rule out chromosomal deletions as the basis of irreversibility may be difficult, but helpful guidelines exist. Deletions involving a given chromosomal locus are usually rare and repeats will not generally be seen unless there is special selective pressure for them. In cases where they do occur, repeat deletions are usually nonidentical in that they are of variable extent. Among organisms carrying plasmids, plasmid-negative variants are usually more common in absolute terms, and repeats are generally identical. Nevertheless, there may be hidden selective influences that enhance the apparent frequency of a deletion and "hot spots" or that limit its extent (see "Deletion Analysis").

With regard to selective and mutagenic effects, it is perhaps not widely recognized that most of the agents or conditions that enhance the appear-

ance of plasmid-negative variants are also mutagenic (254). Further, in some "curing" situations, the positive cell as a whole is more sensitive than the negative to inhibition by the "curing" agent, so that spontaneous negative variants are merely selected rather than produced by the agent. Such selective effects are often due to the presence of specific plasmid-linked determinants of cellular sensitivity to the "curing" agent. Examples are penicillinase-plasmid-linked sensitivity to bismuth and silicate ions (196, 209), F- and R-linked sensitivity to sodium dodecyl sulfate (264), and R(f) and P-lac-linked sensitivity to acridine orange (S. Falkow, *personal communication*; M. Yoshikawa, *personal communication*; see "Epistatic Sensitivity").

A complicated but illustrative case is the enhancement of penicillinase plasmid-negative segregants by novobiocin (R. Novick, *unpublished data*). After overnight growth with a low inhibitory concentration of the drug, 80 to 90% of an originally penicillinase-positive population becomes negative. However, during the first 6 hr there is no increase in the small fraction of negatives initially present, while the viable count falls by a factor of 10-100. Further study (G. Peyru, *personal communication*) has revealed that the rare negatives originally present in the population are very slightly more resistant to novobiocin than are the positives; the negative segregants isolated after growth in the presence of novobiocin are somewhat more resistant, whereas the remaining positives are still sensitive. It appears that the negatives, being slightly more resistant than the positives, can mutate more readily to slightly higher levels of resistance, thus increasing their selective advantage.

Selective sensitivity determinants need not, however, be plasmid-linked. Selection for *E. coli* mutants resistant to coli-phage T1 leads to the isolation of repeated deletions (as well as point mutations) involving a chromosomal locus involved in T1 adsorption (284).

In the classical demonstration of F-curing by acridine orange (114), and in more recent demonstrations of temperature-curing of staphylococcal plasmids (169), possible selective and mutagenic effects were circumvented by determinations of the rate of appearance of negatives during the growth of the positive culture under curing conditions: an F<sup>-</sup> daughter cell was produced in about 50% of cell divisions in the presence of acridine orange (96), and tetracycline- or penicillinase-negative daughters were produced in 75 and 10% of cell divisions at 44 C, respectively (169).

Observations of this type, when coupled with a demonstration that under curing conditions the

negative variants grow at the same rate as their parent strain and that they do not revert, provide virtually unassailable evidence for the segregation of separate hereditary units and for the differential inhibition by acridine or elevated temperature of some essential plasmid functions—presumably replication or distribution.

In cases in which the increase in frequency of negative variants is very small, it is quite difficult to study the kinetics of loss. In such cases it would seem essential to exclude mutagenic and selective effects quite rigorously before accepting the data as evidence for plasmid-linked inheritance. Table 3 shows the results of a number of experiments showing low "curing" frequencies, many of which have not dealt adequately with these problems. In cases in which acridine "cure" frequencies are high, the trait under consideration is spontaneously quite unstable (237). In such cases, mutagenic effects are irrelevant, but a miniscule selective effect could easily produce the observed result.

### Conclusion

The criteria that seem to be unequivocal are those involving linkage, mutations affecting segregation or replication, and isolation of an intact plasmid genome. The others are all more or less circumstantial and should be treated accordingly. Because techniques are now readily available for the isolation of small circular DNA molecules (13, 136, 226), any of the more circumstantial kinds of evidence for plasmid-linked inheritance can be greatly strengthened by the demonstration of a correlation between the phenotype in question and the presence of a specific circular DNA molecule.

Finally, it must be mentioned that these criteria have been established on the basis of the known properties of a number of clearly defined, well differentiated extrachromosomal elements that seem to fit into a definite taxonomic category. There may well be other extrachromosomal elements that do not show clearly all of the properties discussed above. The detection, identification, and characterization of such less well-defined elements, including any not composed of duplex DNA, may require a rather different approach.

### GENERAL DESCRIPTION

Table 4 is a compilation of various extrachromosomal elements that conform generally to the patterns of plasmid organization as described above. Their general properties have been described repeatedly and at length in several excellent reviews (31, 71, 172, 217, 230). Present purposes will be served by a compact summary, including Table 4, which will be found useful for

TABLE 3. Plasmid curing by acridines and temperature

Organism	Marker or plasmid	Minimum no. of cistrons involved in "cure"	Procedure	Results		Author's conclusion: "curing"	Controls done		References
				Rate <sup>a</sup> or frequency of negatives	Enhancement over spontaneous rate or frequency		Reversion of negative to positive	Selective effects of curing agent	
<i>E. coli</i> .....	F	8 <sup>b</sup>	A(K) <sup>c</sup>	50%/generation	500 ×	+	NT	NT	96, 114
<i>E. coli</i> .....	ColV2, I-K94	10	A(ON)	80%/18 hr	80 ×	+	NT	NT	139
<i>E. coli</i> .....	ColV2, I-K94	10	A(ON)	0.3%/18 hr	≥15 ×	+	NT	NT	163
<i>E. coli</i> .....	ColV3-K30	8	A(ON)	0.2%/18 hr	≥10 ×	+	NT	NT	163
<i>Salmonella typhimurium</i> .....	ColI6-P9	9	A(ON)	<0.5%/18 hr	None	—			207
<i>E. coli</i> .....	ColE1	2	A(ON)	<0.1%/18 hr	None	—			163
<i>S. typhimurium</i> ...	ColE2-P9	2	A(ON)	<0.5%/18 hr	None	—			207
<i>E. coli</i> .....	R(t)	11	A(ON)	1-3%/24 hr	10 ×	+	NT	NT	98, 272
<i>Shigella flexneri</i> ..	R(t)	11	A(ON)	50-95%	10 ×	+	NT	NT	98
<i>S. typhimurium</i> ...	R(t) <sup>d</sup>	8	T(K)	10%/generation	≥10 ×	+	NT	NT	262
<i>E. coli</i> .....	K88	1	A(ON)	20%/24 hr	20 ×	+	NT	NT	201
<i>Staphylococcus aureus</i> .....	T <sub>169</sub> <sup>e</sup>	1	T(K)	75%/generation	>100 ×	+	<10 <sup>-11</sup>	none	169
<i>S. aureus</i> .....	P <sub>169</sub> <sup>f</sup>	6	T(K)	10%/generation	>1,000 ×	+	<10 <sup>-4</sup>	none	169
<i>S. aureus</i> .....	P <sup>f</sup>	1	T(ON)	<0.1%-23%/18 hr	None-46 ×	some +	NT	NT	9
<i>S. aureus</i> .....	PI <sub>524</sub>	10	A(ON)	0.1%/18 hr	None	—			189
<i>S. aureus</i> .....	P <sup>f</sup>	11	A(ON)	0.4%-3.5%/18 hr	4-35 ×	+	NT	NT	180

<sup>a</sup> Rates refer to the probability that at any cell division, one daughter is plasmid-negative.

<sup>b</sup> All sex factors are assumed to have at least six cistrons involved in mating; all Col factors have at least one cistron for each colicin and one for resistance to it.

<sup>c</sup> A = acridine dyes; T = growth at elevated temperature; (K) = kinetics of appearance of negative variants; (ON) = culture assayed for negatives after overnight growth under curing conditions; NT = not tested.

<sup>d</sup> Special R factor carrying kanamycin resistance and naturally thermosensitive for stability.

<sup>e</sup> Staphylococcal plasmids are referred to by their salient somatic marker and the strain from which they came.

<sup>f</sup> Composite data from several strains.

reference throughout the rest of the paper. A complete list would be impossible—for example, several hundred different plasmids carrying antibiotic resistance (R factors) have been described. The object here is to single out examples that are sufficiently unique or illustrative to merit separate discussion. In the first column of Table 4 are listed the usual designations of the plasmids in the literature (see Table 1), which generally refer to identifying features of the elements as they are manifested in the host cell phenotype. The notation for colicinogenic factors is that suggested by Fredericq, which includes an indication of the original carrier strain (e.g., Col E1-K30 refers to an E1 colicinogenic factor originally harbored by *E. coli* strain K30). In Table 4 and in the rest of the review, the lack of this notation indicates that complete identification was not made by the author being quoted. Similarly, the notation for staphylococcal plasmids (209) includes the original carrier strain (e.g., PII<sub>147</sub> refers to a penicillinase plasmid of incompatibility type II originally carried by strain 147).

#### Transmissible Plasmids

As the majority of known plasmids are transmissible, the preponderance of this class in Table

4 is not accidental. In addition to F and its derivatives, the class of transmissible plasmids includes F-like fertility factors, some of the Col factors, R(t) factors, and, tentatively, two *E. coli* plasmids involved in virulence. These two, Hly (248) and Ent (249), are transferable agents responsible for alpha-hemolysin and enterotoxin production, respectively, in certain pathogenic strains of *E. coli*. However, it is not clear from the available data (248, 249) whether the transferable complex in either case consists of a single plasmid or of two separate plasmids, one a transfer factor and the other a nontransmissible virulence factor that is frequently cotransferred. Thus, these two are listed only tentatively as transmissible.

Good evidence exists for the unitary nature of the other elements listed as transmissible plasmids in Table 4. Further, of those that have been tested (6, 17, 37, 76, 82, 105, 202, 205, 258), all have sex factor (or fertility factor) activity; i.e., they can mediate the transfer of nontransmissible plasmids and host chromosome fragments. Thus, it seems likely that sex factor activity is a general property of transmissible plasmids.

In addition to determinants of conjugation and equipment for autonomous replication, most transmissible plasmids have been found to affect

visibly their host cell phenotype, carrying genes for characteristics such as colicinogeny, antibiotic resistance, UV resistance, and phage restriction. These genes will be termed "somatic" to distinguish them from those essential for plasmid replication, since the latter do not directly affect the host cell phenotype. Some fertility factors have not been found to carry somatic functions (17, 82, 221). However, this lack is probably due more to a lack of knowledge on our part than to a deficiency of genetic information on the part of the plasmid. The DNA molecule of F, for example, is large enough to code for 50 to 100 different proteins (79), a number that must greatly exceed the requirements of the conjugation system and of autonomous replication. The smallest sex factor of known size, R15 (188), has a molecular weight of 35 million (Table 6), sufficient to code for about 70 different proteins.

The term "fertility factor" is often used to refer specifically to that part of a transmissible plasmid involved in autonomous replication and conjugation. Other somatic determinants are referred to as though they were "attached" or "associated" (76, 139, 186, 270), implying a structural differentiation in addition to a functional one and tending to blur the very real distinction between linked genes carried by a unitary plasmid (270) and an assemblage in a single host of separate plasmids that are often cotransferred (5, 250).

For Col factors, the "association" idea evidently stems from the concept that these plasmids are complicated entities, similar to defective phages and able to carry on a wide variety of autonomous activities, including quiescent and vegetative replication, colicin production and release, and the production of colicin immunity. However, as Herschman and Helinski (107) have pointed out, there are at least two quite different classes of Col factors, namely, colicinogenic sex factors and nontransmissible Col factors such as ColE1; the former seem to be ordinary sex factors carrying the structural gene for a colicin, a gene for resistance (immunity) to it, and perhaps also one for its release, whereas the latter can clearly be induced to replicate vegetatively and thus resemble defective prophages (55).

For R factors, there has been considerable difficulty in working out the linkage arrangement of resistance genes in a multiply resistant organism. It is often difficult to decide how many separate extrachromosomal linkage groups are involved and how the different markers are arranged within them. Part of this difficulty stems from the possibility that some R factors may be assemblages of two or more independent replicons that reversibly dissociate (188; see Fig. 15c).

For some time there has been considerable

disagreement between Watanabe and co-workers and Anderson and co-workers on the genetic structure of R(t) factors; the former have maintained that R(t) factors are unitary (270, 275), whereas the latter have suggested that they are assemblages of nontransmissible resistance plasmids in conjunction with a transfer factor which mobilizes them (4, 5). As strong evidence has been presented by both groups, the conclusion seems inescapable that both situations commonly exist and should be accepted as such. In this context, an important question is whether there is any evolutionary relationship between unitary and separated R factors. A further complication of R factor genetics is that many strains harbor pairs of compatible R(t) factors (221, 260) or an unmarked sex factor in conjunction with an R(t) factor (221) so that, unless a careful attempt is made to separate different cotransferable linkage groups, a very misleading picture can emerge (221).

#### Nontransmissible Plasmids

There is a rather heterogeneous group of extrachromosomal elements that cannot bring about their own transfer; many of these elements, however, can be transferred in association with a sex factor. Included in this group are a number of colicinogenic factors and R(nt) factors, as well as a plasmid carrying the K88 antigen determinant (202) and several cryptic plasmids, that become manifest when they have incorporated a segment of host genome (3, 236).

The staphylococcal plasmids (189, 192, 195, 209) belong to this collection, as there is nothing except their host to distinguish them from other nontransmissible R factors. The question of conjugal transfer of staphylococcal plasmids is academic since mating has not been demonstrated for their host organism.

Phages such as P1, whose prophage is an autonomous replicon (20, 126) are also included in this group as are phages such as f1, which establishes a nonlethal infection where mature particles are continually released from infected cells that continue to grow (118).

#### Cryptic Plasmids

In this section are considered a diverse group of elements whose somatic functions have not been identified. Such plasmids have manifested themselves in two ways: (i) by having incorporated a specific chromosomal fragment which behaves differently in its extrachromosomal state than in its chromosomal state (*pi* factors), or (ii) by having DNA that can be separated from that of its host's chromosome. Also considered in this section are the question of essential plasmids and

TABLE 4. Properties of extensively studied plasmids<sup>a</sup>

Plasmid	Typical or usual host	Best evidence for ECI or AR <sup>a</sup>	Vegetative or lethal replication	Infective particles	UV or MC inducibility	Sex factor activity	Sensitivity to male-specific phages		Fertility repressor (specificity)	Integration into chromosome	Curable by		References
							fd	id			Acridines	Temp	
F, F' F <sub>φ</sub> <sup>+</sup> , WG-4, etc.	<i>E. coli</i>	Non-linkage Transmissibility	— (—)	—	— (—)	+	+	—	— +	+	+		17, 82, 201; Clowes, pers. comm. <sup>b</sup> 119, 158
	<i>E. coli</i>								+	—	—		
FP Δ F <sub>φ</sub> -lac ColV2, I-K94 ColV3-K30 ColB, V-K260 ColV-CA7	<i>Pseudomonas aeruginosa</i>	Transmissibility		—		+							5 12, 68 139, 163 139, 163 75, 186, P. Cooper, pers. comm. R. Clowes, pers. comm. R. Clowes, pers. comm. R. Clowes, pers. comm.
	<i>Salmonella</i>	Transmissibility	(—) (—)	—	(—) (—)	+	+	+	+	(—) (—)	—		
	<i>Salmonella</i>	F incompatibility	—	—	—	+	+	—	—	(+)	+		
	<i>E. coli</i>	F incompatibility	—	—	—	+	+	—	—	(+)	+		
	<i>E. coli</i>	F incompatibility	—	—	—	+	+	—	—	+	+		
	<i>E. coli</i>	F incompatibility	—	—	—	+	+	—	—		+		
	<i>E. coli</i>	Transmissibility	—	—	—	+	+	—	+	(F)	—		
ColB1-CA18 ColB2	<i>E. coli</i>	R-incompatibility	—	—	—	+	+	—	+	(F)	—		R. Clowes, pers. comm. R. Clowes, pers. comm.
	<i>E. coli</i>	Transmissibility	—	—	—	+	+	—	+	(F)	—		
ColB3-K177	<i>E. coli</i>	Transmissibility	—	—	—	+	+	—	+	(F)	—		R. Clowes, pers. comm.

[illegible]

\* Abbreviations not in Table 1: ECI = extrachromosomal inheritance; AR = autonomous replication; MC = mitomycin C; fd = DNA-containing, phage-specific for F-like sex pili; id = DNA containing, phage-specific for I-like sex pili; (-) or (+) = probably - or +; -? or +? = information in literature is contradictory, - or + is the best guess.  
<sup>b</sup> Personal communication.

<sup>b</sup> Personal communication.

TABLE 5. *Autonomy spectrum*

	Quiescent autonomous replication	Synchrony with cell cycle*	Conjugal transmissibility	Vegetative replication	Lethal replication	Free infective particles
R(nt)	+	+	—	—	—	—
F	+	+	+	—	—	—
		(—)				
ColIb-P9	+	+	+	(—)	(—)	—
		(—)				
R(t)	+	+	+	+	±	—
		(—)				
ColE1-K30	+	+	—?	+	+	—
		(—)				
fd	+	+	—	+	—	+
P1	+	+	—	+	+	+
		(—)				
λ	—	+	—	+	+	+
		(—)				

\* Parenthesis indicates that under certain conditions, the element multiplies faster than the host chromosome.

the genetic basis of certain cases of instability that have been attributed to "controlling episomes" (53, 112).

**Pi factors.** Ames et al. (3) examined a series of unstable histidinol-utilizing revertants of *hisG203*, a strongly polar deletion of the operator end of the histidine operon in *Salmonella typhimurium*. These revertants were characterized by: (i) a reduplication of the histidine operon except for gene *G* (the reduplicated segment is termed *pi*); (ii) apparent attachment of *pi* to a cryptic plasmid (the use of *pi* is expanded to include other cryptic plasmid that have incorporated chromosome segments. These composite elements will be referred to as "*pi*-factors"; e.g., the histidine plasmid would be *pi-his*.); (iii) frequent segregational loss of *pi* with restoration of the original *hisG203* genotype; and (iv) restoration of function of the reduplicated genes now not under repressive control by histidine.

The author's (3) interpretation of the restoration of function is that the histidine operon deprived of its operator-promoter end must be fused to some other controlling system, evidently one borne by the cryptic plasmid. Functional connection between unrelated operons has been demonstrated clearly in *E. coli* for the connection of tryptophan genes to an operon of the transducing phage,  $\phi 80$  (229), and for the connection of lactose genes to a purine operon (133) or to the tryptophan operon (14). In all three cases, the affected genes are no longer under their usual

regulatory control and, in the latter two cases, the *lac* genes have been shown to be controlled by adenine and by tryptophan, respectively.

The instability of *pi-his* could be the result of replacement by the *his* region of a semiessential region of the original cryptic plasmid. [Instability resulting from deletion of part of an R factor has been described (175).] In that case, the entire plasmid should be missing from *pi*<sup>−</sup> segregants. Alternatively, the *pi*-factor could lose its *his* region, either by a reversal of the process by which it acquired *his*, by deletion, or by nonreciprocal recombination with the chromosomal *his* region.

In a second *pi*-factor, described by Schwartz (235, 237, 238), a duplication of a section of the *E. coli* chromosome carrying the *ilv met* region as well as a suppressor (*sup*) for a particular *lacZ* allele has become attached to a genetic structure shown in conjugational crosses to be unlinked to the chromosome and therefore evidently a cryptic plasmid. The phenotype is unstable Lac<sup>+</sup>, which reverts either to the original Lac<sup>−</sup> through loss of the duplicated fragment or to stable Lac<sup>+</sup> through integration of the suppressor locus into the chromosome.

Since *pi* factors appeared only after EMS treatment, it is not unlikely that multiple events were involved in their formation: duplication of a chromosomal region, including a suppressor locus; mutation of one copy of the suppressor locus; and attachment of the duplicated region to a cryptic plasmid. If the suppression were due to an alteration of the coding specificity of a transfer ribonucleic acid species, duplication could be necessary to preserve viability.

An additional feature of this system is that secondary Lac<sup>−</sup> derivatives could be induced by EMS to give once again unstable *sup* revertants. Therefore, the Lac<sup>−</sup> derivatives must retain at least one copy of the cryptic plasmid. If so, either the Lac<sup>−</sup> segregants result from loss by the *pi*-factor of the chromosome segment, or there are at least two copies of the plasmid, one of which remains cryptic and is retained when the unstable copy carrying the chromosome fragment is lost. The possibility of two genetically different variants of the same plasmid existing more or less stably in the same cell raises the problem of the usual incompatibility barrier to this situation, but there is at present insufficient information to warrant further discussion.

A third *pi*-factor has been described, involving the tryptophan region of *S. typhimurium* (164; P. Margolin, *personal communication*). This *pi-trp* factor is quite similar to the *pi-his* factor, and its identification strengthens the concept of *pi*-factors as a distinct class of bacterial plasmids.

**Cryptic plasmid DNA.** At least four different occurrences of plasmid-like DNA in bacteria have recently been described. Rownd et al. (224) found in an *E. coli* strain a minor DNA component with a base ratio of 64% guanine plus cytosine (G + C); Cozzarelli et al. (43) found, in a series of *E. coli* strain 15 derivatives, a circular species of DNA with a molecular weight of  $1.45 \times 10^6$ ; Lee and Davidson (155) isolated circular DNA of molecular weight 0.88 million from *Micrococcus lysodeikticus*; and Rush (*personal communication*) found in a *Shigella* strain no less than six different species of circular DNA with molecular weights ranging from 1.0 to 24 million (see Fig. 4b). None of these DNA species has yet been correlated with any host phenotype. Thus they all represent, at least temporarily, cryptic plasmids.

**Controlling episomes.** Unstable reversions from auxotrophy to prototrophy have also been studied by Hill (112) and by Dawson and Smith-Keary (53). These reversions were all characterized by segregation of variants similar or identical to the original auxotroph. In the studies of Dawson and Smith-Keary, additional observations were interpreted as suggesting that instability "migrated" or was "transposed" from one site to another (53) and that the location of a point mutation, *proB401*, varied within the *proB* cistron (123).

To explain these observations, Dawson and Smith-Keary suggested that a novel type of extrachromosomal element was involved, a "controlling episome." This controlling episome was envisioned as being able to interact with the chromosome in a variety of ways, based on its reversible "attachment" to chromosomal sites. Any number of similar or different controlling episomes could be present in a cell; attachment could be highly specific for given chromosomal sites, or could show very little specificity, the element migrating freely from one site to another. On attaching, the controlling episome could prevent function of the gene to which it was attached or induce permanent chromosomal mutations (in either of two directions) at that site, or both. Since the results of Hill and those of Dawson and Smith-Keary have been widely quoted as evidence for this "controlling episome" model of genetic variation and instability, it seems worthwhile to point out that the available data are not really adequate to establish the model and that key experiments to test its predictions have not been reported.

In examining a case of apparent instability, one must first decide whether the instability is truly genotypic and, if so, whether it can be accounted for by a heterozygous duplication. The likelihood

of duplications accounting for instability is especially great in the case of suppressors. As discussed by Hill et al. (111), where suppression requires mutational alteration of the coding specificity of an essential tRNA species, the organism may need to retain a wild-type allele of the corresponding tRNA cistron; thus there may be strong selective pressure for a heterozygous duplication involving that cistron.

Duplicated segments are known to be unstable if they are *cis* and tandem to their original, or *trans* and part of a *pi* factor. Tandem duplications are inherently unstable (32, 111, 121) because of the possibility of forming excision loops according to the Campbell model (31); the resulting crossover eliminates one set of the duplicated genes (Fig. 2). *Pi* factors are also unstable, for reasons that are not yet clear.

In two studies of unstable reversions of auxotrophs (53, 112), the observed results could be accounted for by the occurrence of heterozygous duplications of either type. In a third paper, that of Smith-Keary and Dawson (251), in which unstable *Pro*<sup>+</sup> revertants of a proline auxotroph were described, it was not clear whether the instability was hereditary; the preponderance of auxotrophic cells in revertant colonies could have been accounted for by slow-growing revertants feeding the surrounding cells. Again, the possibility of heterozygous duplications was not dealt with. Further, one series of experiments was interpreted as consistent with the view that the "controlling episome" "migrated" within the

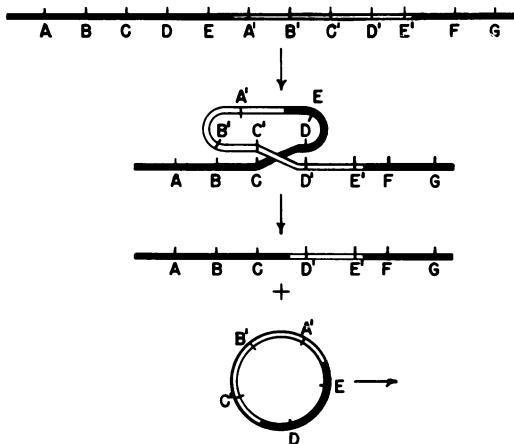


FIG. 2. Excision of tandem duplication. A-G refer to loci. A'-E' refer to duplicated loci; a loop brings homologous C-D and C'-D' regions into register. A single reciprocal crossover restores continuity of chromosome now carrying only one copy of each locus and releases nonviable fragment.

*proB* locus, producing point mutational changes at different sites within that locus. This interpretation predicts that different sublines carrying point mutations at different sites should produce *proB* gene products with different single amino acid replacements. No study of these gene products has been reported. Study of two stable *Pro*<sup>-</sup> sublines of *proB401* revealed the production of prototrophic recombinants in crosses between them (123). The map positions of the two *Pro*<sup>-</sup> mutations were also shown to be different by crosses with other (stable) *proB* mutants. These findings are uninterpretable by any known genetic mechanism and the authors' interpretation, transposition of a "controlling episome," is not very enlightening. Unless a number of other similar cases occur, this observation will probably end up as one of the unsolved but intriguing curiosities that are encountered in genetics from time to time. If, on the other hand, other similar occurrences can be documented and shown clearly to be due to insertions and transpositions of episomes, then the implications for microbial genetics will be very far reaching.

At the moment, however, the "controlling episome" theory must be taken as no more than an interesting speculation and one that must be tested very carefully in any given instance, since, with its nearly magical powers, it provides a facile explanation for a wide variety of peculiar genetic results, an explanation that one may be tempted to accept without critical experimental support.

#### Essential Plasmids

Plasmids carrying functions essential to the survival of their host would be cryptic in the sense that one could not identify them directly by comparison of plasmid-positive and plasmid-negative strains. One could attempt to identify them by looking for conditional lethal mutations whose wild-type alleles behaved during transfer to mutant strains as though they were plasmid-linked. The F merogenote, F-13, is an essential plasmid in its original host strain, having incorporated a segment of host chromosome and leaving behind a corresponding deletion, including essential genes. Elimination of F-13 from this strain with acridine orange is apparently lethal. Treatment with acridine orange selects for cells in which the entire F-13 element has become integrated into an aberrant site of the chromosome, producing a new Hfr derivative with translocated genes and therefore different transfer characteristics from the original Ffr-13 (232). On transfer to other strains, F-13 behaves like an ordinary F merogenote. Aside from plasmids carrying es-

sential genes which have been deleted from the chromosome, no other essential plasmids have been discovered to date.

#### Certain Phages

Most temperate phages would not be considered extrachromosomal elements as defined above since, in their autonomous state, they are not stable components of the cell genome. However, the dividing line between plasmids and phages is by no means sharp, so that it will be instructive to consider briefly some of the phages in terms of their solutions to the replication control problem as well as in terms of their contrast to the plasmids (Fig. 2).

#### Extrachromosomal Inheritance in Other Organisms

Extrachromosomal elements of one sort or another have been identified or suspected in the *Neisseria* (137), *Vibrio* (18), and *Serratia* (15). There is some evidence for the occurrence of transformational heterozygotes in pneumococcus (127); whether this heterozygosity involves extrachromosomal elements remains to be seen. There has been considerable discussion of the possibility that certain genes related to sporogenesis in some of the gram-positive bacteria are, in fact, extrachromosomal (132, 220), but again, concrete evidence is lacking.

There is a growing number of examples in a variety of organisms of genetic traits that appear to become lost irreversibly at low frequencies. In some cases the rate of this loss appears to be increased by growth in the presence of acridine dyes. At least some of these traits are doubtless plasmid-linked, but I feel there is not sufficient evidence in any of them to warrant a definite conclusion.

### CLASSIFICATION

#### Transmissible Plasmids

As conjugal transmission involves a complex and highly evolved genetic structure that is evidently shared by all transmissible plasmids [with the possible exception of FP (119, 158), which has not yet been studied in detail], it seems likely that these elements bear a close evolutionary relationship to one another (172, 271) and therefore comprise a taxonomic entity.

Transmissible plasmids of the *Enterobacteriaceae* can be divided into two distinct and non-overlapping subsets on the basis of sex pilus structure (150, 172). Two types of sex pili have been identified (150) and are referred to as F-pili and I-pili, respectively, according to whether they resemble pili produced by one prototype

sex factor, F, or the other, ColIb-P9 (150, 172). The two types of pili are morphologically and serologically distinct and differ in their specificity as receptors for male-specific phages (150). F-pili adsorb spherical (e.g., f2, QB, MS2) and filamentous (e.g., f1, fd) F-specific phages but do not adsorb I-specific phages (e.g., If1, If2); the converse is true of I-pili (150). A large number of plasmids have been examined for sex pilus specificity (150, 170, 221) and each has been found to control the production of either F-pili or I-pili. No intermediate types have been identified, nor has any single plasmid been found to produce both types.

Most sex factors have determinants for repression of their own fertility (66, 182), the effect of which is to prevent or drastically reduce the formation of sex pili (187). Mutant sex factors (*drd*) and certain wild-type sex factors, notably F, lack fertility repressors and in consequence have greatly enhanced fertility as well as sensitivity to male-specific phages (171). Because certain combinations of sex factors producing the same type of pilus are compatible, it has been possible to show that fertility repression is dominant over derepression and that, in general, repressed sex factors of one type inhibit pilus formation by derepressed plasmids of the same type but not by those of the other type (172, 198, 221, 274, 276).

Watanabe et al. (276) initiated the practice of referring to R factors (and other sex factors) that repressed the fertility of F as  $f^+$  and to those that did not as  $f^-$ . Since both  $f^+$  and  $f^-$  sex factors have turned out to have active fertility repressors, the notations  $f^+$  and  $f^-$  are somewhat misleading. Derepressed wild and mutant plasmids of both pilus types would also be listed as  $f^-$ . Since the taxonomic differentiation of the two types is actually based on pilus specificity rather than on repression, Meynell et al. (172) have adopted the expedient of referring to the  $f^+$  and  $f^-$  classes of Watanabe et al. (276) as F-like and I-like, respectively. I suggest a shorthand notation for these two groups in which (f) or (i) would be appended to the usual designation of a plasmid to specify whether it was F-like or I-like. Thus,  $R(f^+)$  would be R(f) and  $R(f^-)$  would be R(i). Derepressed mutants would be  $R(fdrd)$  and  $R(idrd)$ , respectively. Examples of exceptions to these groupings are  $F_{\phi-lac}$ , a derepressed plasmid whose sex pili adsorb filamentous F-specific phages but not spherical ones (150), and R(i)62, a plasmid that directs the synthesis of I-like pili but represses the fertility of F as well as its own fertility (221).  $F_{\phi-lac}$  is probably a genetic variant of an F-like

plasmid with respect to pilus structure; R(i)62 is likely to be a recombinant plasmid (172) with an intact I-fertility determinant and a heterozygous duplication of at least that part of an F-fertility region containing the locus for fertility repression (see Fig. 11).

A secondary criterion of evolutionary relatedness is that of incompatibility, i.e., inability to cohabit a single cell stably (see "Incompatibility"). As was previously discussed (209), the genetic mechanism underlying compatibility and incompatibility among plasmids is complex, highly evolved, genetically stable, and therefore taxonomically significant. Further, incompatibility appears to be a general property of pairs of isogenic plasmids (i.e., isogenic except for point mutational differences in scorable markers). Consequently, incompatibility is taken to be an indication of evolutionary proximity; the converse, compatibility, is taken to be an indication of some evolutionary distance. Thus, the highest order of generic differentiation shown in Fig. 1 is compatibility relationships, indicated along the bottom line, where each vertical represents a group of mutually incompatible plasmids. Such groups will be referred to as incompatibility sets. Although only a small number of the possible combinations of plasmid pairs have been tested for incompatibility, it has been generally true that independently isolated plasmids are compatible and therefore belong to separate incompatibility sets. Because compatibility is the general rule, Fig. 1 places different plasmids in separate incompatibility sets unless the contrary is known to be true, as for example with F and the ColV factors (140, 163).

One confirmation of this classification scheme is that all but one of the known naturally occurring derepressed plasmids are elements of the same incompatibility set. This set includes all four of the ColV factors for which sufficient data are available. One of these, ColV-CA7, is nontransmissible, but because it manifests entry exclusion toward F (see "Interactions Between Replicons"), it is probably a defective F-like sex factor (P. Cooper, *personal communication*). In the F-ColV set are the only sex factors that have been found to give rise to Hfr and F-merogenotes (76, 138) and also the only ones that show significant acridine curability in *E. coli* (139). This acridine sensitivity is not the result of the derepressed state of these sex factors, since *drd* mutants of R(f) factors are no more sensitive to acridine curing than are their repressed parents (Y. Hirota, *personal communication*).

The R(f) factors all apparently belong to a single incompatibility set as do the R(i) factors

(E. Meynell, *personal communication*). No example of incompatibility between an R(f) and an R(i) has been encountered. In the case of R factors, therefore, incompatibility and pilus specificity coincide (178, 198, 276). Known examples of incompatibility between an R factor and another sex factor include R(f) and certain ColB factors (R. Clowes, *personal communication*) and R(i) and ColI (E. Meynell, *personal communication*). Since all sex factors that belong to an incompatibility set also belong to the same pilus specificity class, whereas all elements that control the production of one type of pilus do not belong to a single incompatibility set, it is clear that the pilus-specific classes can each be divided into a series of unique and nonoverlapping incompatibility subsets, as has been done for Fig. 1.

Other possible criteria of relatedness are transfer competition and recombination. In a cell harboring F and  $F_0$ -lac or Hfr and  $F_0$ -lac, F-mediated transfer is normal but  $F_0$ -lac transfer is suppressed by a factor of  $10^8$  (68). This suppression is clearly not due to fertility repression (unless F has a very peculiar repressor that acts only in *trans*) but may be due to competition for transfer initiation sites. I have not found any data on similar experiments for other sex factors.

Recombination between nonhomologous plasmids is usually rare, sporadic, and difficult to quantitate. Recombination between the following pairs of nonhomologous plasmids has been observed: R(f)  $\times$  F (99, 277);  $F_0$ -lac  $\times$  P1 (68); R(f)  $\times$  P1 (147); P1  $\times$  PII (195); ColV, B-K260  $\times$  F-lac, and ColV, B-K260  $\times$  R(f) (P. Fredericq, Ciba Found. Symp., *in press*). Possibly a systematic analysis of these kinds of events would reveal definite relationships (see "Recombination Between Heterogenic Plasmids").

It is probable that exceptions to this classification scheme will occur. If recombination between nonhomologous elements should happen to result in an exchange between fertility and compatibility determinants (which are unquestionably separate), the result might be, for example, an R(i) factor carrying the compatibility determinant of an R(f) and thus belonging to the R(f) incompatibility subset.

#### Nontransmissible Plasmids

The nontransmissible plasmids are a diverse collection and are grouped merely for convenience. They are relatively few and their diversity is underlined by the lack of any incompatible pairs among separately isolated elements. A possible exception to the overall classification is ColK-K235, which is evidently incompatible with an R(t) factor (142). Fredericq (76) stated

that ColK-K235 is not a sex factor, whereas Meynell et al. (172) stated that it is; neither presented evidence. If ColK is not an overt sex factor, it could owe its incompatibility with R(t) to derivation from one.

The nontransmissible factors have been divided into two groups on the basis of whether they multiply vegetatively with possible lethal consequences for the host cell. The vegetative class comprises inducible but nontransmissible Col factors, along with four phages which have been included for illustrative purposes. Coliphage P1 should perhaps be included with the inducible Col factors, since it seems to resemble them in being autonomous in its quiescent stage (see "Control of Replication"). Among the nonvegetative plasmids are R(nt) factors, *pi* factors, and the K88 plasmid.

#### Staphylococcal Plasmids

A series of nontransmissible plasmids have been identified in *S. aureus*. All so far identified carry antibiotic resistance markers and may be considered nontransmissible R factors. It is not known whether there is any genetic or evolution-

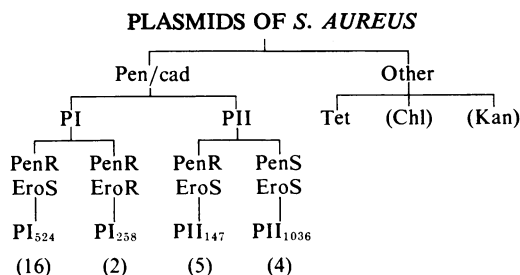


FIG. 3. Classification of plasmids harbored by *S. aureus*. Pen/cad refers to a series of plasmids that are grossly homologous and were termed penicillinase plasmids (195) because penicillinase production was a salient plasmid-linked characteristic. Plasmids obviously belonging to the series but lacking the penicillinase gene (209) have prompted the inclusion of cad as a generic character, because resistance to cadmium is one of two markers invariably present. PI and PII represent the two incompatibility sets within which a further subdivision has been made on the basis of penicillin and erythromycin resistance. Nomenclature is according to Peyru et al (209); a subscript refers to the naturally occurring strain in which a plasmid was originally found. In parentheses are numbers of separately isolated plasmids with the indicated marker patterns. Within each group a variety of different patterns involving the other markers occur (see Fig. 9). Other plasmids carry resistance genes for different antibiotics; no additional markers for these plasmids have been identified. Parentheses indicate uncertainty about extrachromosomal status.

TABLE 6. *Plasmid-specific DNA*

Plasmid	Host	Contour lengths <sup>a</sup>	Monomer mol wt (in millions of daltons)	Total DNA <sup>b</sup>	Most probable no. of copies per chromosome	References
		$\mu m$		%		
ColE1	<i>Proteus</i>	2.3, 4.7, 6.9	4.5	0.2-0.3	4	90, 222
ColE1 <sup>c</sup>	<i>Proteus</i>		4.5	4-10	20-50	55
ColV, B, <i>cys</i> , <i>try</i>	<i>Proteus</i>	54.5	107	5	1	110
F	<i>E. coli</i>		45	2	1	79
F- <i>lac</i>	<i>E. coli</i>		74			79
F'2	<i>E. coli</i>		81			80
F- <i>gal</i>	<i>E. coli</i>		51			80
F- <i>gal att</i> $\lambda$	<i>E. coli</i>		72			80
F- <i>gal att</i> $\lambda(\lambda_1)$	<i>E. coli</i>		105			80
F- <i>gal att</i> $\lambda(\lambda_1\lambda_2)$	<i>E. coli</i>		138			80
R(f)222	<i>Proteus</i>	7, 28, 35	69			188
R(i)15	<i>Proteus</i>	18	35			188
P1 phage	—	37	72			126
P1 prophage	<i>E. coli</i>	32	63			126
$\lambda dv$	<i>E. coli</i>		8	6	60	167
$\phi X174(RF)$	<i>E. coli</i>	1.6, 3.2, 4.6, 6.0	3.1			226
15 plasmid	<i>E. coli</i> 15	3.0	1.45	0.8	12-15	43
PI <sub>258</sub>	<i>S. aureus</i>	9.4	18.8			225
PI <sub>258-ero16</sub> <sup>d</sup>	<i>S. aureus</i>	8.2	16.3			225
PI <sub>258-pen102</sub> <sup>d</sup>	<i>S. aureus</i>	7.5	15.2			225

<sup>a</sup> Mostly measurements of open circular forms seen in the electron microscope.

<sup>b</sup> Estimates of total amount of plasmid DNA per chromosome.

<sup>c</sup> After mitomycin C induction.

<sup>d</sup> Deletions; see Fig. 9.

ary relationship between the staphylococcal plasmids and the enteric R factors, although resistance to many of the same drugs is involved in both.

The staphylococcal plasmids fall into several groups. The best studied are: the penicillinase plasmids (189, 192, 195, 209), which occur in a homologous series with a variety of marker patterns (209) including determinants for penicillinase production and control (189); resistance to erythromycin (103); and resistance to a series of inorganic ions, namely mercury (218), arsenite, arsenate, cadmium and lead (192), and bismuth (209). Penicillinase plasmids form two incompatibility sets (195, 209), as do the R(t) factors; the region of the plasmid responsible for compatibility has been identified and roughly localized (192). Tetracycline resistance seems to be plasmid-borne in some strains (8, 169, 210), and there is suggestive evidence that chloramphenicol resistance and kanamycin resistance are also plasmid-borne (33). The latter three resistance determinants are linked neither to one another nor to penicillinase production and are probably

all mutually compatible. These relationships are shown in Fig. 3, in which the major divisions represent incompatibility sets.

#### Autonomy and Infectivity

The typical plasmids and other more or less plasmidlike endosymbionts listed in Table 5 show a spectrum of behavior with respect to two separate but related activities, namely autonomy and infectivity.

At one end of the spectrum are the benign, relatively quiescent R(nt) factors; at the other end are those temperate phages that are always lethal in their autonomous state. The R(nt) factors are manifested by their resistance determinants; their replicative autonomy has no special impact on their host organism. Though dispensable, they are genetically stable; spontaneous loss in many cases occurs at a rate of  $10^{-3}$ /cell generation or less (189, 214). Incompatibility studies (see "Basis of Incompatibility") and studies with segregation mutants (130, 193) suggest that there are not more than one or two copies per chromosome. Thus there must be

rather strict coordination between plasmid replication and the cell division cycle.

Sex factors such as F are also present in one or two copies per chromosome (130), are closely coordinated with chromosome replication and segregation (49), and therefore have a degree of autonomy similar to that of R(nt) factors. In addition, they are transmissible; of necessity, during their epidemic spread among an uninfected population, they multiply faster than the host chromosome. At least one transmissible R-factor, when harbored by *Proteus*, appears to have the capacity for a somewhat greater degree of replicative autonomy. In exponential cultures, R-DNA amounts to about 12% of the total, or about 12 copies per chromosome (224). As the culture enters stationary phase, R replication continues until R-DNA accounts for at least one third of the total (223). Thus, the R factor is capable of replicating either more frequently, as frequently, or less frequently than the host chromosome. After transfer, which involves presumably a single copy, it must replicate more frequently until a complement of 12 copies is achieved. During subsequent exponential growth, it remains a constant fraction of the cell genome and therefore, on the average, each copy replicates as frequently as the chromosome. On emerging from stationary phase, the R factors must replicate less frequently than the chromosome, until their number has been reduced to the steady-state level.

A study of the overall replication pattern of the plasmid population (223) has shown that plasmid units replicate at random. During any one cell division cycle, some plasmid DNA will have replicated twice or more, some once and some not at all (223). This situation suggests poor, if any, coordination of plasmid replication with the cell division cycle and permits a good deal of flexibility in plasmid replication frequency. What controls the frequency of plasmid replication relative to that of the chromosome is unknown. Perhaps intercellular heterogeneity in frequencies of R factor replication is involved: most of the R factors could be quiescent while those in a few cells were rampant. With some R factors (in *Proteus*), lysis of about 25% of the cells is observed as the culture enters stationary phase (S. Falkow, Ciba Found. Symp., *in press*). Whether this partial lysis is related to population heterogeneity with respect to plasmid replication and is to be considered in the same light as cell death from vegetative phage or Col factor replication is not certain.

ColIb-P9, a transmissible plasmid, seems to have the same degree of autonomy as the other sex factors. It is included in Table 5 because it

has been reported to be capable of lethal vegetative replication (1, 2, 183). However, these reports are controversial.

Col E1-K30 is representative of a class of plasmids that are evidently a step closer to the phages. It can be induced to replicate vegetatively (55) with lethal consequences for its host (107, 206), but is probably not transmissible (139). However, it seems unlikely that ColE1 kills by unrestricted replication, since vegetative plasmid replication per se is not necessarily lethal. Matsuura (167) has described a coliphage lambda fragment ( $\lambda dv$ ) that replicates extensively (60 copies per cell) without obvious damage to its host (see Table 6). Host killing by ColE1 could be due to the colicin itself (206) or to some other substance induced concomitantly. A report that colicin E1 production was *not* the lethal event because cells induced in the presence of chloramphenicol were killed nevertheless (107) is inconclusive. Viability determinations required removal of the inhibitor, possibly allowing resumption of colicin synthesis.

The filamentous male-specific phages are a curious combination of phagelike and plasmid-like characteristics. Although their vegetative replication is very similar to that of the virulent single-stranded DNA phages such as  $\phi$ X174 and S13 (165a), they do not kill the host, but establish a stable infective state where the cell extrudes mature phage particles while continuing to grow and divide (118). It is likely, as is the case with  $\phi$ X174, that these phages have a unique replicative form that establishes itself at a specific site in the cell, prevents superinfection by homologous phages, and produces progeny RF that cannot themselves replicate. Thus, it seems to become a permanent extrachromosomal component of the cell genome. The fate of the unique RF in dividing cells is, however, unclear (165a). Is it inherited unilinearly or is it transmitted plasmidlike, one copy to each daughter cell? These filamentous phages are morphologically very similar to sex pili (23) and have been likened whimsically to sex factors that carry their genome within their mating organ rather than within their host bacterial cell.

Coliphage P1 is near the end of the spectrum, since its lethal vegetative state results in a crop of infectious particles. It evidently has evolved a system of control of quiescent autonomous replication, since it is autonomous in its prophage state as well as in its vegetative state (20, 126). Coliphage lambda, at the end of the spectrum, is unable to control its autonomous state, which is therefore always lethal. Its solution to the control problem for prophage replication is to

allow the host chromosomal replicon to take over via integration of the prophage. These points are summarized in Table 5, which illustrates the continuum.

#### PHYSICAL BASIS OF EXTRACHROMOSOMAL INHERITANCE

DNA corresponding to a number of different plasmids has been isolated by various methods from plasmid-positive cells, characterized physicochemically and in some cases examined in the electron microscope (13, 55, 79, 80, 110, 126, 188, 222, 225). Without exception, intact DNA of established plasmids has been found to be in the closed circular duplex configuration. Usually this DNA has a base composition similar or identical to that of the chromosome in its natural host; advantage has often been taken of the transferability of plasmids from *E. coli* or *Salmonella* to genera such as *Proteus*, which has a different DNA base composition, to permit direct pycnographic isolation of their DNA (72). Other isolation procedures include that of the Freifelders (78), who found conditions where only F DNA replicates and incorporates radioisotopes, whereas Ikeda and Tomizawa (126) and Bazaral and Helinski (13) took advantage of the differential binding of the intercalating dye, ethidium bromide, to superhelical as compared with linear or open circular DNA (44, 211) to isolate the autonomous P1 prophage genome and the ColE1 plasmid, respectively, from *E. coli*. A different property of closed circular duplex DNA, that of its selective renaturation following alkali denaturation and neutralization of whole cell lysates, was used by Rush et al. (225) to isolate penicillinase plasmid DNA from *S. aureus* and cryptic plasmid DNA from *S. flexnerii* Y6R (Fig. 4). Some of the properties of isolated DNA of various plasmids are listed in Table 6.

The correlation between genetic data and physical measurements for F-*gal* and several of its derivatives (80), and for penicillinase plasmid PI<sub>258</sub> and two of its deletions (225), strongly supports the identification of circular DNA molecules with the respective extrachromosomal elements (see Fig. 10).

Where relevant data are available, results of physical studies are generally in conformity with the genetic data indicative of no more than one or two copies of a plasmid per chromosome. Exceptions to this generalization are *Proteus* carrying ColE1, which replicates vegetatively after mitomycin C treatment, and *Proteus* carrying an R factor, of which there are evidently multiple copies during normal exponential growth. Isolation of multiple species of circular DNA from certain R(f)<sup>+</sup> strains (188; S. Falkow,

Ciba Found. Symp., *in press*) suggests that some R(f) factors may be composed of multiple replicons that reversibly associate and dissociate (see Table 5 and "Model for Recombination Between Isogenic, Incompatible Plasmids"). This possibility could account for the frequent loss of markers observed with such R(f) factors (270; T. Watanabe, Ciba Found. Symp., *in press*). Conversely, an R(i) factor that does not show marker instability was found to consist of but a single DNA species (188).

Still somewhat obscure is the physical basis for most of the bacteriocins. Among the colicinogenic factors whose DNA has been isolated, namely ColB, V-K260 (110), ColE1 (55), and ColE2 and E3 (13), all belong to the class that produces soluble colicins. Others of this class, namely ColI, ColB, and other ColV factors, are clearly plasmids by other criteria. Among the class of colicinogenic factors that produce particulate, phage-related colicins (see review by Bradley, 23) and are considered to be defective prophages, ColK-K235 appears to be plasmid-linked by virtue of its apparent incompatibility with an R factor (142), whereas the determinant of the phagelike particle (67), colicin 15, appears to be integrated into its host's chromosome. A series of derivatives of *E. coli* strain 15, some colicinogenic, some not, were examined by Cozzarelli et al. (43), and all were found to contain molecules of circular DNA with a molecular weight of  $1.45 \times 10^6$ . As no other circular DNA species was encountered, it seems unlikely that extrachromosomal circular DNA is responsible for production of the colicin. If DNA corresponding to this colicinogenic factor as well as that responsible for other phagelike bacteriocins is integrated in its quiescent state and therefore not detectable, it should be detectable during induced production of the corresponding bacteriocin, insofar as the factor resembles a defective prophage.

#### SOMATIC FUNCTIONS: MECHANISMS AND CONTROL

I suggest, as a working hypothesis, that genes found to be plasmid-linked in wild-type organisms owe their plasmid linkage to a selective advantage that such linkage confers. This hypothesis involves two possible alternatives: (i) plasmid-borne determinants may have evolved as such in a current host or (ii) they may have been introduced from some other host where they had evolved as chromosomal genes and had subsequently become plasmid-borne in the manner of F-merogenote formation (128). If the former is true, then the evolutionary advantage of the plasmid state can be inferred from the de facto

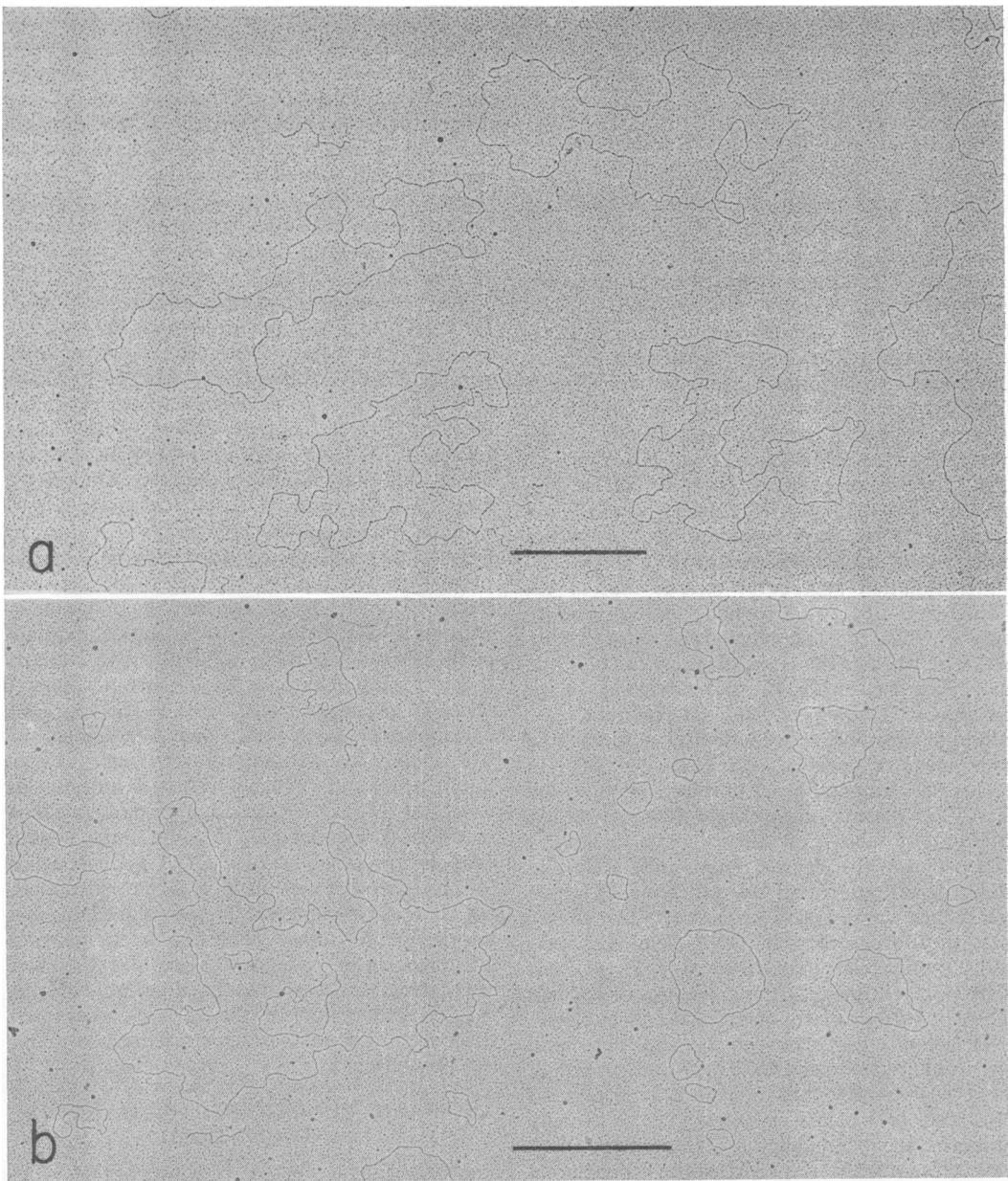


FIG. 4. Electron micrographs of plasmid DNA prepared from (a) *S. aureus* and (b) *S. flexnerii* Y6R by Mark Rush. Bar indicates one micron. The staphylococcal preparation is of  $PI_{258}$  (see Fig. 9) and is monodispersed. The *Shigella* preparation is of cryptic plasmids and shows five of the six different sized species of circular DNA found (225). The photographs were kindly supplied by Charles Gordon.

situation. If the latter is true (see "Integration of plasmids"), then the evolutionary advantage of the plasmid state is a matter of necessity. Lacking the requisite homology for an integrating cross-over, the cell must harbor the plasmid or lose the gene. An instructive example of this situation

is the plasmid-borne *lac* determinants found in *Salmonella* ( $F_0$ -*lac*) (68) and *Proteus* (P-*lac*) (73), in which neither organism seems able to provide itself with a functioning chromosomal *lac* complex and so has occasionally accepted a foreign, plasmid-borne one in preference to none. Lack-

TABLE 7. *Plasmid somatic functions*

Marker <sup>a</sup>	Phenotype	Action	Carried by	References
<i>fer</i> <i>eex</i> <i>pil</i> <i>col</i>	Conjugal transmissibility Surface exclusion Male-specific phage sensitivity Colicinogeny	Mating complex Antigen? Sex pili	Sex factors Sex factors Sex factors Col factors, R(i) factors	172
<i>cor</i>	Colicin resistance	Unknown	Col factors, R(i) factors	75, 113
<i>spp</i> <i>res</i>	Phage resistance Phage restriction	Unknown Nuclease	F P1, R(i) factors, other I-like sex factors	97, 233, 286 93, 113, 154, 276
<i>mod</i>	Phage modification	Unknown	P1, R(i) factors, other I-like sex factors	113, 154
<i>uvr</i>	UV resistance	Unknown	ColI, ColB, R(i), R(f)	57, 122, 183
<i>asa</i>	Arsenate resistance	Unknown	P plasmids	192, 196
<i>asi</i>	Arsenite resistance	Unknown	P plasmids	192, 196
<i>bis</i>	Bismuth resistance or epistatic sensitivity (Bi <sup>+++</sup> )	Unknown	P plasmids	196
<i>lea</i>	Lead resistance (Pb <sup>++</sup> )	Unknown	P plasmids	192, 196
<i>cad</i>	Cadmium resistance (Cd <sup>++</sup> )	Unknown	P plasmids	192, 196
<i>mer</i>	Mercury resistance (Hg <sup>++</sup> )	Unknown	P plasmids, R factors	192, 218
<i>cob</i>	Cobalt resistance (Co <sup>++</sup> )	Unknown	R factors	246
<i>nic</i>	Nickel resistance (Ni <sup>++</sup> )	Unknown	R factors	246
<i>sil</i>	Silicate sensitivity	Unknown	P plasmids	209
<i>sds</i>	Dodecyl sulfate sensitivity	Unknown	F, R factors	264
<i>acr</i>	Acridine sensitivity	Unknown	P-lac, R(f)	S. Falkow, <i>pers. com.</i> ; M. Yoshi- kawa, <i>pers.</i> <i>com.</i>
<i>penI</i>	Penicillinase control	Unknown	P plasmids	189
<i>penB</i>	Penicillinase control	Unknown	P plasmids	215
<i>penZ</i>	Penicillin resistance	$\beta$ -Lactamase	P plasmids, R factors	51, 189
<i>ero</i>	Erythromycin resistance	Unknown	P plasmids	103
<i>chl</i>	Chloramphenicol resistance	Acetylase (+AcCoA) <sup>c</sup>	R factors <sup>e</sup>	33, 176, 200
<i>str</i>	Streptomycin resistance <sup>b</sup>	Adenylase (+ATP) <sup>d</sup>	R factors	200, 268
<i>str</i>	Streptomycin resistance	Phosphorylase (+ATP)	R factors	266
<i>kan</i>	Kanamycin resistance	Acetylase (+AcCoA)	R factors <sup>e</sup>	33, 200, 267
<i>kan</i>	Kanamycin resistance	Phosphorylase (+ATP)	R factors <sup>e</sup>	200, 266
<i>neo</i>	Neomycin resistance	Phosphorylase	R factors	266, 278
<i>sul</i>	Sulfonamide resistance	Unknown	R factors	177
<i>tet</i>	Tetracycline resistance	Unknown	R factors <sup>e</sup>	169, 177
<i>hly</i>	Alpha-hemolysin production		Hly factor	248
<i>ent</i>	Enterotoxin production		Ent factor	249
<i>K88</i>	K88 surface antigen production		K88 factor	202

<sup>a</sup> These designations are in accord with the proposals of Demerec et al. (54) on nomenclature, except that in certain cases the marker is known to represent multiple cistrons (e.g., *fer*, *pil*); in other cases, the marker may represent more than one cistron.

<sup>b</sup> With the aminoglycoside antibiotics, it is not certain whether a given inactivating enzyme is specific for a single compound or general for the group.

<sup>c</sup> Ac CoA = acetyl coenzyme A.

<sup>d</sup> ATP = adenosine triphosphate.

<sup>e</sup> R factors, other than P plasmids, carrying the marker are found in *S. aureus* as well as in coli-form bacteria. For staphylococcus, the only markers whose action is known are *chl* and *penZ*.

ing homology with the chromosome, the plasmid-borne *lac* determinants must remain so.

Not knowing the requisite evolutionary history of the various plasmid-linked genes that would enable me to do otherwise, I have simply listed as plasmid somatic functions (Table 7) those determinants that are plasmid-linked in naturally occurring strains. Most of these genes have not been found to occur naturally as chromosomal loci. Although there is no way of deciding definitely on the origins of those that have, a strong circumstantial case can sometimes be made, as in the case of *lac*, for a chromosomal origin.

Among plasmid somatic functions are some that directly subserve the purposes of the plasmid itself vis à vis its survival as an endosymbiont, namely, fertility and integration functions. Others, e.g., resistance determinants, seem more directly to subserve the economy of the host cell. The latter may be considered, in a sense, as an evolutionary remuneration that the plasmid pays to its host.

### Fertility

Enteric bacteria harboring transmissible plasmids produce, under plasmid control, specialized pili (sex pili) required for genetic transfer (26) and used as adsorption sites by male-specific phages (25). Plasmid-linked fertility functions may also include mechanisms for initiating DNA transfer.

The complexity of these functions has only recently become apparent through analysis of fertility-defective mutants, of which the vast majority are plasmid-linked (50, 203), suggesting a major role for the plasmid in the genetic determination of fertility. Fimbriae, as distinct from sex pili, are host-determined (162) and are also involved in mating, having been found to stabilize mating pairs (185). Fimbriation deficiency is the only form of host-determined infertility (185, 234) thus far described.

Fertility-deficient F-factor mutants show a variety of patterns of resistance and sensitivity to the different male-specific phages. Thus, those mutants that fail to make sex pili are fully resistant to all male-specific phages; others make normal-looking pili but are nevertheless resistant; still others are sensitive to some phages (QB, f1) but resistant to others, (f2, MS2) and (242, 243). In the latter case, phages f2 and MS2 adsorb to the pili but their RNA does not enter the cell. A fourth phenotypic class is fully sensitive to all male-specific phages but nevertheless defective in mating (203). In this latter class, either the pili are subtly defective or there is a defect in an unknown stage of genetic transfer that does not

involve the pili. Plasmid-linked genes required specifically for chromosome transfer have not been identified by mutation: all known fertility-defective mutants are equally deficient in chromosome transfer and in sex-factor transfer. Chromosome mutations that impair chromosome but not sex-factor transfer include *recA* mutations (36, 39) and others whose effect on recombination is not clear (115).

Fertility-defective mutants of R(f)100 have also been isolated (115) and, since the F and R plasmids are compatible, complementation studies have been feasible. The result of these studies was the identification by Otsubo (203) of at least six and possibly as many as nine cistrons involved in the genetic control of fertility. A set of fertility-defective mutants of F-*gal* were mapped by deletions affecting the F-linked galactose operon as well as the fertility region (see Fig. 10). No fertility-defective mutation has been found to impair the capacity of a sex factor for autonomous replication; therefore, the fertility system has been included among somatic functions rather than among essential ones. The role of DNA replication in genetic transfer has been the subject of some controversy. If replication is required, then some replication-specific functions may also be fertility functions.

### Recombination, Integration, and Immunity

Among the vegetative functions of phages such as  $\lambda$ , are several that some of the more typical plasmids may also have; for example, those involved in recombination, including integration into the host chromosome, and those involved in superinfection immunity. Since the recombination functions are not directly manifested in the host phenotype, they are not, strictly speaking, somatic functions. They are mentioned here because they would presumably not be essential for plasmid autonomy either; they have not, in fact, been demonstrated directly for any of the plasmids.

Superinfection immunity is a property of plasmids, but its mechanism seems different from that of prophage immunity; as it is probably related to the essential plasmid functions, it will be discussed later in this paper (see Incompatibility).

### Bacteriocins

Among the bacteriocins, only colicins are known definitely to be produced by extrachromosomal elements and only pneumocins (173) are known definitely not to be. The large class of bacteriocins that resemble phage structures are determined by elements that seem similar to defective prophages (see review by Bradley, 23), but no such element has yet been identified.

The plasmid-linked colicins are macromolecules of various degrees of complexity ranging from relatively simple proteins (108) to complex lipoglycoproteins (89, 124); they include at least one example of a phagelike particle, a component of colicin K-K235 (23). In general, colicins adsorb to specific cell membrane sites and exert their lethal action through interruption of vital cellular processes (see review by Luria, 160). Colicin determinants are doubtless complex and may involve a number of different genes, including structural cistrons for the colicin itself whose number will depend upon its complexity, genes for the control of colicin synthesis (198) and perhaps for its release, and at least one gene for colicin resistance ("immunity"). Genetic analysis of colicin determinants has yet to be initiated.

#### Resistance to Physical, Chemical, and Biological Agents

The largest recognized class of extrachromosomal genes in bacteria are those concerned with resistance, including resistance to antibiotics, colicins, inorganic ions, UV light, and bacteriophages. As with fertility determinants, proof that resistance genes are not essential rests on the isolation of plasmid deletions or mutations that do not impair plasmid autonomy. In the case of the staphylococcal plasmids, this object has been accomplished for each of the known resistance markers (192). Deletions of R factor-linked resistance genes have also been observed to be without effect on autonomy (270).

Because most of the things to which plasmids confer resistance act by attacking vital cellular target functions and plasmids probably do not alter these basic functions, it follows that plasmid-linked resistance determinants must erect "accessibility barriers" (191). These barriers may involve binding or enzymatic inactivation of a toxic substance or a decrease in permeability of the cell or a subcellular compartment. If permeability barriers are involved, plasmids must alter at least some of the properties of the cell membrane; in this connection, it is perhaps noteworthy that  $F^+$  and  $F^-$  organisms have been found to differ in a number of surface properties [see Freifelder (77) for a discussion of these differences].

Resistance to colicins deserves special comment because of its common association with colicinogeny. There are several mechanisms of plasmid-linked resistance to colicins. One involves loss of the specific receptor site for the colicin; others involve some step in lethal colicin action subsequent to adsorption. Receptor-site colicin resistance, carried by certain non-colicinogenic R factors (T. Watanabe, *personal communica-*

*tion*), may involve more than one type of colicin as well as certain phages. Postadsorption resistance has been found in connection with both colicinogenic and non-colicinogenic plasmids (240). In the former case, it is referred to as "immunity," because it is specific for the colicin produced and has been assumed to be an inevitable consequence of colicinogeny. The apparent similarity between specific immunity conferred by prophages and specific resistance conferred by Col factors led to the prevalent notion that the two situations are analogous. Clearly, however, this is not the case. Prophage immunity is due to a genomic repressor of phage reproduction, whereas colicin "immunity" is resistance to the immediate lethal action of the protein itself. Col factors capable of vegetative replication must have replication repressors and doubtless would confer immunity against vegetative replication of superinfecting homologues. However, one must distinguish this true immunity from the specific colicin resistance that must have evolved as a necessary defense against the lethal action of the colicin always present in populations of Col<sup>+</sup> bacteria.

#### Epistatic Sensitivity

Some plasmids carry determinants that increase the sensitivity of their hosts to certain substances. Such sensitivity markers must be "epistatic" rather than "dominant" to the natural resistance of the host for these substances, since plasmid genes are not generally alleles of chromosomal loci. An important example is the sensitivity to male-specific phages of cells harboring sex factors (156). Other epistatic plasmid-linked determinants of sensitivity have been identified in staphylococcus and in *Enterobacteriaceae*.

Like plasmid-linked resistance, epistatic sensitivity often involves accessibility barriers. This is obviously the case for phage adsorption by sex pili and probably the case for acridine, SDS, and bismuth sensitivity; although bismuth uptake does not appear to be increased by epistatic sensitivity determinants (G. Peyru, *personal communication*), there may be some sort of internal accessibility barrier that is lowered by them.

#### Phage and UV Resistance

Many sex factors and other plasmids carry determinants of phage resistance (154, 286) which often involves DNA restriction-modification. Resistance determinants for phages tau (97) and T3, of which the latter is a restricting system (233), are the only somatic functions aside from fertility known for  $F$ . Phage restriction is also the only known somatic function of sex factor  $\Delta$  (5). Many of the R(i) factors have restricting

systems (11), in one case associated with a definite nuclease distinct from known host nucleases and from the usual host restricting-modifying systems (259, 271).

Likewise, a number of sex factors, including ColIb-P9 (122, 184), ColB1 (122), R(i) (56), and R(f) (57), have been found to carry determinants of UV resistance distinct from the known *uvr* loci of the host bacteria. Mechanisms of plasmid-linked UV resistance are unknown.

### Converting and Hft Phages

Phage conversion is a form of extrachromosomal inheritance in which the phenotype of a bacterial cell is modified by the presence of a prophage. Modifications of this sort may occur by either of two general mechanisms. (i) The prophage modifies the expression of a particular chromosomal gene by always integrating in or near it or by producing some substance that controls its function or modifies its product; (ii) the prophage itself carries the structural gene for some determinant of host phenotype. Such genes are generally expressed during vegetative phage growth as well as in lysogens (265). I am unable to quote any well-documented example of (i). In (ii), the converting gene may be the structural gene for the phage repressor, leading to immunity toward superinfecting homologous phages, or to other effects such as restriction of T4rII mutants by  $\lambda$  (H. Eisen, *personal communication*). Alternatively, it may be a gene that has no apparent relationship to phage function and is not subject to the genomic repression of the prophage. Examples in the latter category are DNA-restricting or -modifying systems (or both), and the structural gene for a somatic antigen (24, 157). The converting phage,  $\epsilon^{15}$  of *Salmonella*, carries the structural gene for an enzyme that replaces a host enzyme involved in the synthesis of O-antigen and, in addition, produces an inhibitor of the "action" of that host enzyme. Converting phages that carry structural genes are indistinguishable from nondefective Hft phages, and one wonders whether both originate by the same mechanism (*see* "Recombination Between Heterogenic Plasmids"). How converting genes escape repressor control of the entire phage genome (64), i.e., how they escape fusion to a phage operon, is difficult to understand. In the case of  $\phi 80dtrp$ , incorporated *trp* genes do appear to be under phage repressor control (229).

### Other Somatic Functions

Plasmids carrying various other genetic determinants have been identified. Among these are the two *lac* plasmids mentioned above and

others carrying determinants of a soluble alpha-hemolysin (the Hly factor, 248), an enterotoxin (the Ent factor, 249), and a surface antigen (the K88 factor, 202). The alpha-hemolysin determinant was transmissible in 10 of 53 hemolysin-positive strains initially tested by Smith and Halls (248). A similar transfer frequency was found for enterotoxin production (249). Whether these determinants are chromosomal in strains unable to transmit them is not known. In strains able to transmit the K88 antigen by conjugation, the antigenic determinant is evidently borne by a nontransmissible plasmid, accompanied by an unlinked sex factor of F specificity (202).

### Structural Genes

Although there is no reason to suspect that the plasmid-linked genes discussed here are not, in fact, the structural loci for the gene products involved, it seems worth mentioning that, with the exception of F-merogenotes, the plasmid location of a structural gene has been rigorously established in only a single case, namely the penicillinase locus in *S. aureus*. This proof consisted in isolating a structural penicillinase mutation, transferring the mutant plasmid to a new host, and showing that the same structural mutation involving the gene product was cotransferred (M. Richmond, *personal communication*). Less rigorous data are available for other plasmid-linked resistance markers and fertility markers in which point mutations are cotransferred with the plasmid (50, 102, 196, 203). In these cases, the possibility has not been rigorously excluded that the structural genes for the traits involved are actually chromosomal and that the observed plasmid-linked determinants are required for their expression. The converse situation, an unlinked gene that controls the expression of a specific plasmid locus, has recently been identified by Cohen and Sweeney (42), who found that a class of mutations to constitutive penicillinase production were not linked to the plasmid and superseded the usual control of the plasmid-linked penicillinase region, which remained genotypically inducible. Other nonplasmid-linked mutations that prevented the expression of particular plasmid-linked resistance genes have been observed in our laboratory (196).

### Control of Plasmid Somatic Functions

Genes regulating operon function can be differentiated into two classes on the basis of their behavior in complementation tests. Repressor-producing genes are usually dominant to their mutant alleles in the *trans* position, whereas operators and promoters are unaffected by their

alleles in the *trans* position and are therefore *cis* dominant (131). It is not certain that all genes belong to operons; the demonstration of inducibility or repressibility or the isolation of *trans*-recessive control mutations may be taken as *prima facie* evidence that a gene is part of an operon.

Coliphage lambda possesses a number of separate operons, but there is one repressor locus, *C<sub>r</sub>*, that directly or indirectly controls the function of the entire phage genome (*see* 64). This type of control has been termed "genomic repression" to distinguish it from control systems involving only one operon. It is relevant to the relation between plasmids and phages to ask whether plasmids are controlled by genomic repressors or whether their highest level of regulatory organization is that of the independent operon. In fact, both types of organization evidently occur among the plasmids.

Determinants of penicillinase (86, 189) and of arsenate resistance (196) carried by the same plasmid in *S. aureus* are independently inducible by their substrates and therefore belong to independent operons. Plasmid-linked constitutive mutations have been isolated for penicillinase (189) and shown to be *trans* recessive (213). Chloramphenicol acetylase, linked to a different staphylococcal plasmid, is also inducible (181); however, in *E. coli*, R factor-linked penicillinase (52) and chloramphenicol acetylase (W. Shaw, *personal communication*) are not substrate-inducible. Inducibility has not been reported for other plasmid somatic functions such as UV resistance, phage restriction, and resistance to antibiotics other than the ones just mentioned. These functions seem to be effectively expressed throughout a population of plasmid-positive cells. The fertility system in most sex factors is under *trans* dominant repressive control; thus, the set of fertility cistrons comprises one or more operons. Moreover, these cistrons appear to be clustered (203). Though independent plasmid-borne operons are clearly demonstrable, there is no evidence for UV-induced genomic derepression of sex factors and other quiescent plasmids. Thus, UV does not stimulate transfer of either fertility-repressed sex factors (271) or of fertility-derepressed ones (105, 283). The unlikely possibility remains that these plasmids have genomic repressors that are released by a stimulus as yet undiscovered.

An understanding of the control of colicin production has been retarded by the tendency to consider all Col factors as defective phages. Recently, a few clarifying observations have emerged. Production of the lethal substance

itself appears to be under fertility-repressor control in some colicinogenic sex factors but not in others, where it is under separate control of its own. Thus, in R(i)144-ColIb, *drd* mutations affecting fertility markedly increased the production of colicin Ib (65), whereas in ColIb-P9, *drd* mutations had no effect on colicin production, and a mutation resulting in thermal induction of colicin production had no effect on fertility (198). Similarly, production of ColV, carried by a derepressed sex factor, is not repressed by an R(f) factor that does repress ColV transfer (R. Clowes, *personal communication*). There is no information on control of levels of "immunity" or colicin release, nor is there evidence bearing on fertility-repressor control of other plasmid-linked genes. UV inducibility of colicin I production is doubtful (*see* "Control of Replication"). There is no good evidence for genomic repression of ColI factors.

It is possible that hyper-replication of R factors in *Proteus* is due to release from a mild form of genomic repression—hypersynthesis of R-linked drug-inactivating enzymes and a certain amount of cell lysis occur concomitantly (S. Falkow, Ciba Found. Symp., *in press*). However, hypersynthesis could simply be the result of high gene dosage. With clearly inducible plasmids, such as ColE1 (55), the case for genomic repression is much better; presumably, repression of colicinogeny is released by UV irradiation concomitantly with derepression of vegetative replication.

## INTERACTIONS BETWEEN REPLICONS

Conjugal matings between two F<sup>+</sup> strains are very inefficient by comparison with matings between an F<sup>+</sup> and an F<sup>-</sup> strain (152). This inefficiency, involving chromosomal gene transfer as well as sex-factor transfer has been loosely termed "superinfection immunity" (270, 276), implying an analogy with prophage immunity. I would like to suggest that this analogy is spurious, as is the analogy between colicin "immunity" and prophage immunity, and that the observed inefficiency can be understood as a combination of two quite different effects, namely "entry exclusion" and "plasmid incompatibility."

### Entry Exclusion

The following observations attest to the existence of a sex-factor-linked determinant of entry exclusion (*eex*) that imposes a barrier to the physical transfer of DNA between cells carrying isogenic or closely related sex factors. This barrier is sex-factor-specific—if a donor harbors

two sex factors and a recipient harbors one of them, the exclusion applies only to DNA transfer mediated by the one *both* strains carry. Exclusion is evidently independent of sex piliation and plasmid incompatibility; it probably involves a specific alteration of the cell surface.

(i)  $F'$  DNA is transferred to DNA-less mini-cells derived from an  $F^-$  parent but not to those derived from an  $F^+$  parent, despite the absence of  $F$ -DNA and male pili in the latter (41).

(ii) ColII DNA is transferred from HFCT donors to Col<sup>-</sup> recipients but not to ColII<sup>+</sup> recipients (M. Monk, *personal communication*) despite stringent repression of pilus formation by the recipient Col factor.

(iii) Crosses between a strain harboring an  $F$  factor and one harboring an  $R(f)$  factor are fertile in both directions (274), despite the production by both cell types of similar or identical sex pili (150, 187).

(iv) Whereas conjugal transfer of an  $R(f)$  factor is inhibited by an  $R(f)$  factor in the recipient strain, no such inhibition is seen if the donor plasmid is transferred by transduction (279; S. Mitsuhashi, *personal communication*).

(v)  $R$  factors have been isolated that show no entry exclusion toward other  $R$  factors yet are active as donors (T. Watanabe, Ciba Found. Symp., *in press*). Conversely, cells harboring fertility-defective mutant sex factors, unable to produce sex pili, are still infertile as recipients (203).

(vi) Experimental evidence for or against fertility repressor control of *eex* is not available. Entry exclusion is, however, associated with sex pilus specificity. Though members of one sex pilus class do not all show entry exclusion toward one another, all sex factors that do exclude one another are of the same type with respect to sex piliation (65, 172, 198, 276).

(vii) Entry exclusion inhibits transfer from Hfr donors as strongly as from  $F^+$  donors, although the chromosome segments transferred from Hfr donors are not incompatible with the resident sex factor.

(viii) Entry exclusion can be abolished by alterations in phenotypic properties of the recipient cell either by growth to stationary phase (152) or by treatment with periodate (252), neither of which affect the cell *genotype* nor facilitate the establishment of a superinfecting sex factor (60).

### Incompatibility

Plasmid incompatibility rather than entry exclusion is the basis of certain cases of interference between isogenic or closely related sex factors.

Thus, *F-lac* cannot stably superinfect an Hfr strain even if the entry barrier is removed (60); ColV3-K30 cannot stably superinfect  $F^+$  *E. coli*, although  $F$  does not manifest entry exclusion toward this Col factor (163).

Entry exclusion and incompatibility are distinctly different phenomena, but they are often associated; elements that show entry exclusion toward one another are often in the same incompatibility set.

In bacteria, incompatibility appears to be a fundamental property of replicons in that it is universally exhibited by plasmids isogenic for autonomy functions. Thus, various  $F$ -merogenotes are incompatible with one another (46, 63, 231) as are derivatives of an  $R(f)$  factor (102), a Col factor (186, 198), and a staphylococcal plasmid (192, 194, 195).

If a single basic mechanism accounts for all cases of incompatibility, then it must account for the rather different incompatibility patterns seen with different plasmids, as discussed below. (In this discussion, "establishment" refers to the process of becoming a stably inherited component of the cell genome.)

(i) Plasmid incompatibility is seen in crosses between isogenic strains and therefore does not involve DNA restriction; thus, an entering plasmid is not destroyed by a resident plasmid with which it is incompatible but is merely delayed or prevented from becoming established.

(ii) This delay may vary within wide limits. *F-lac*, after entering an Hfr strain, can be induced for  $\beta$ -galactosidase for at least seven generations but is unable to multiply and never gets established if care is taken to prevent integration by recombination (60); *F-lac* entering an *F-gal* strain, however, has a very good chance of establishing (96). A staphylococcal plasmid,  $PI_{258}$ , can eventually establish itself in nearly 100% of  $PI$ -harboring cells into which it is transduced, although its establishment in some transductant clones appears to take as long as 20 generations (R. Novick, *unpublished data*).

(iii) In general, establishment seems to involve the eventual segregation of a superinfecting incompatible plasmid into a separate cell line. A recently superinfected cell harboring two incompatible plasmids has three alternatives on division: it can give rise to two heterozygous daughter cells, a heterozygous one and a homozygous one, or two homozygous ones. The relative probabilities with which these different events occur seem to vary widely in different systems (96, 195, 276), although it is difficult to be certain, as quantitative data are rarely available.

(iv) The entering plasmid is often on a footing

very different from that of the resident one. In *S. aureus*, plasmid transductant clones isolated without selection for a donor plasmid marker are composed of a very small fraction of donor-type cells ( $10^{-2}$  to  $10^{-5}$ ) and a large majority (the remainder) of recipient types (R. Novick, unpublished data). Thus, the entering plasmid appears unable to multiply while awaiting an opportunity to become established. The resident plasmid, meanwhile, evidently continues to multiply at its normal rate. Thus, cell divisions producing one heterozygous and one homozygous daughter seem to be the most frequent in this situation.

(v) Different incompatible but nonisogenic sex factors have been found to show a hierarchy of preference over one another for establishment. As mentioned, ColV3-K30 is unable to superinfect an  $F^+$  strain, and  $F$  cannot superinfect a ColV2-K94 strain. Conversely, ColV2-K94 can very efficiently establish itself in an  $F^+$  strain, as can  $F$  in a ColV3-K30 strain (163). In the latter two cases, clones derived from exconjugant recipients are usually pure or nearly pure for the donor plasmid. In the former two cases, the donor sex factor cannot ordinarily be detected in clones derived from recipient exconjugants, although a compatible donor plasmid, ColE1, is readily transferred and established. Moreover, ColV2-K94 can efficiently superinfect an Hfr strain and establish itself stably alongside the integrated sex factor (163).

**Basis of incompatibility.** Available evidence suggests that  $F$  factors (131), penicillinase plasmids (195), and other quiescent replicons are represented by no more than one or two copies per chromosome. To ensure the hereditary stability of these nonessential replicons, the cell must provide specifically for equitable distribution of their replicas during cell division. A plausible explanation of this situation is that of Jacob et al. (130), who suggested that every autonomous replicon is attached to a structural component of the cell, a "maintenance site," and that this attachment is necessary not only for distribution of replicas but also for replication itself. Immediately before replication is scheduled to begin, a new site is synthesized; attachment of one replica to the new site is part of the initiation process; the two sites subsequently grow apart, thereby enforcing the segregation of replicas. It is noteworthy that for certain cytoplasmically inherited lambda derivatives, which evidently lack an extrachromosomal maintenance site (285), even as many as 60 copies per cell are not sufficient to ensure hereditary stability (167).

The universal occurrence of incompatibility between pairs of isogenic plasmids suggests the

obvious prediction that, if the maintenance site model is correct, the sites must be replicon-specific. Each plasmid would then have a corresponding locus responsible for specific maintenance site attachment; compatible plasmids would differ in their attachment specificity and would be matched to different maintenance sites, whereas incompatible ones would have the same attachment specificity and would compete for a single site.

According to the maintenance site model, the explanation for the finding that an incoming plasmid is disadvantaged with respect to a resident incompatible one is that the former is unable to replicate until it becomes properly attached. If its site is occupied, it resides unreplicated in the cytoplasm until an opportunity for attachment becomes available; the state of heterozygosity involving two incompatible plasmids will thus be transmitted unilinearly to progeny until segregation occurs. It follows from this model that plasmids of a particular host organism can be divided into groups on the basis of incompatibility; a group of mutually incompatible plasmids comprises an "incompatibility set"; if two plasmids are each incompatible with a third, then they must be incompatible with each other. Incompatibility sets are nonoverlapping; each member of any one set is compatible with each member of every other.

This theoretically limitless array of plasmids and incompatibility sets has at least the following biological limitations.

(i) The number of possible incompatibility sets is large but finite, being related to the number of different base sequences that can be accommodated by that stretch of plasmid genome responsible for maintenance site specificity.

(ii) This number is also physically limited by the number of different maintenance sites a particular organism can offer.

(iii) There may be other limits on the number of different plasmids that a cell may support. For example, in large numbers they may tax the resources of the cell or its available volume sufficiently to give plasmid-negative variants a growth advantage. Combinations of four or five different plasmids in a cell seem to be stable (62, 101). Larger numbers have not so far been examined.

#### Evidence for the maintenance site model

**The segregation unit.** Evidence for stable physical association of the  $F$  factor with a structural component of the cell has been provided by Cuzin and Jacob (49) who showed that a mutant  $F$ -*lac* thermosensitive for replication (*seg*<sup>-</sup>) continues to associate with the same

original chromosomal DNA strand during at least seven generations of growth at a temperature where the mutant F factor is unable to replicate. Chromosomal integration of the F factor cannot account for these results, since the chromosome continues to replicate, whereas the F factor apparently fails to do so. Thus, a plasmid and one strand of a chromosome must have been attached permanently to a common cellular structure, a "segregation unit," that retained its integrity during cell multiplication.

A number of observations support the thesis that bacterial chromosomes are attached to the cell membrane or mesosome (83, 148, 228, 247). If so, then it must be presumed that plasmids are similarly attached (130).

**Host mutants.** The maintenance site model entails specificity on the part of the host in the provision of sites and therefore predicts the occurrence of host mutants defective for maintenance of plasmids belonging to one incompatibility set only. Such mutants have, indeed, been isolated. Staphylococcal mutants thermosensitive for maintenance of type PI plasmids (see Fig. 3) were able to maintain normally those of type PII (193; R. Novick, *unpublished data*); *E. coli* mutants thermosensitive for F maintenance (130) were able to maintain normally R(f) and R(i) (116; Y. Hirota, *personal communication*). Note that the occurrence of these mutants bespeaks "maintenance" specificity but says nothing about "sites"; the host cell could conceivably provide "cytoplasmic" substances specific for maintenance of plasmids belonging to a given incompatibility set. Thus, the connection between maintenance mutations, the segregation unit, and attachment sites remains hypothetical albeit likely.

**Plasmid mcr region.** Results with staphylococcal plasmids have demonstrated a locus for incompatibility specificity. Each of 44 recombinants from a cross between a pair of compatible elements was found to have the incompatibility specificity of one of the parental plasmids. Further, to the limits of resolution afforded by this experiment, a determinant of maintenance site specificity cosegregated with the incompatibility locus. Each of the recombinants was tested for maintenance in a mutant host unable to maintain type PI. All those that were incompatibility type PI were not maintained, whereas those of incompatibility type PII were (192). The maintenance site model predicts that the plasmid region involved in maintenance site attachment is necessary for autonomous plasmid replication. Again in the staphylococcal system, deletions involving this region have always resulted in loss of plasmid

autonomy (192). The deletion map has low resolution so that one may not conclude that a single locus is involved, only a local region.

**Incompatibility sets.** Consistent with the role of discrete plasmid-site interactions in the determination of incompatibility is the finding that incompatibility sets are discontinuous. For example, each of thirty staphylococcal plasmids examined could be assigned unequivocally to either of two sets. There were no intermediate types nor were there single elements capable of interacting with both sites. Thus, plasmid-site interactions appear to have evolved to a high degree of specificity (209).

### Complicating factors

**F/Hfr incompatibility.** According to the maintenance site model, the inability of F to super-infect Hfr means that F occupies its specific site whether or not it is integrated. If initiation of replication is contingent upon site occupation, then the replication of Hfr chromosomes seems to contain a paradox (R. Pritchard, *Heredity*, *in press*), compounded by the ability of F to integrate with either polarity. How is the chromosome to initiate both at the F site and at the chromosomal site and to end up with any semblance of a completed round of replication? Among the possible explanations of this paradox are the following.

(i) The Hfr chromosome occupies only the F site and not the original chromosome site. This possibility predicts that a second, F<sup>-</sup> chromosome, would be compatible with the Hfr one.

(ii) Both sites are occupied but, on initiation of replication at one site, a signal is automatically transmitted that shuts off potential initiation at other possible sites in the *cis* configuration. This possibility predicts that whichever replicon normally initiates first would be the controlling one in the tandem situation.

**ColV plasmids.** The behavior of the two ColV factors with respect to each other and to F (163) suggests that, whereas isogenic plasmids must have the same affinity for their maintenance site, incompatible but nonisogenic ones may have quite different affinities. Note that, in order to become established, an incoming plasmid need not physically displace a resident one from its maintenance site; it need only preempt the new site. When one plasmid is integrated (Hfr), it cannot be preempted by an isogenic entering one (F); evidently a preemptive element (ColV2-K94) must have a greater affinity. The apparent inability of F to replicate in Hfr strains suggests that there is only one F site per chromosome. The ability of ColV2-K94 to establish in Hfr

strains without impairing their fertility (163) suggests that the integrated F need not be attached and that attachment is not required for transfer. This suggestion is supported by the observation that Hfr chromosomes with two separately integrated F factors are stable and can initiate transfer at either of the two corresponding origins (34).

**Persistent heterozygosity.** In its simplest and most stringent form, the maintenance site model involves a plasmid-to-chromosome ratio of one, so that during the cell division cycle the plasmid number parallels the chromosome number (this situation will be referred to as a  $1 \rightleftharpoons 2$  cycle). Incompatibility in an unmodified  $1 \rightleftharpoons 2$  cycle should be very strict; heterozygosity involving two incompatible plasmids should be inherited only unilinearly.

As has been seen, incompatibility in many systems seems less stringent; a heterozygous cell is able, with a probability dependent on the system, to produce two heterozygous daughters. For example, although incompatibility in *S. aureus* is fairly stringent and transductant clones in incompatible crosses are usually pure when selected for a donor marker, when selection is for a donor and a recipient marker jointly, unstable persistent heterozygotes can be isolated (see "Results with Staphylococcal Plasmids").

The incompatibility between R(f) factors (102, 276) and that between F' factors (96) seems even less stringent. In incompatible crosses involving either R(f) or F' factors, clones selected for a donor plasmid marker are nearly always heterozygous and the heterozygosity, though unstable, can usually be maintained by selection. Stable clones with two autonomous incompatible plasmids of any type have not been isolated.

Four possibilities may be considered to account for persistent heterozygosity involving incompatible plasmids.

(i) Associative recombination occurs, and the resulting double plasmid occupies the single available attachment site; being a tandem duplication, the double is unstable; dissociation occurs, and the incompatible products segregate. This possibility is testable with recombination-deficient hosts.

(ii) The plasmid cycle is not  $1 \rightleftharpoons 2$  but some multiple, such as  $2 \rightleftharpoons 4$ . If so, an additional assumption is necessary to account for persistent heterozygosity, namely random segregation of replicas with respect to one another (Fig. 5). In a  $2 \rightleftharpoons 4$  cycle, if both replicas of one plasmid were always apportioned to the same daughter cell, the effect would be the same as in a strict  $1 \rightleftharpoons 2$  cycle; if one replica of each plasmid were

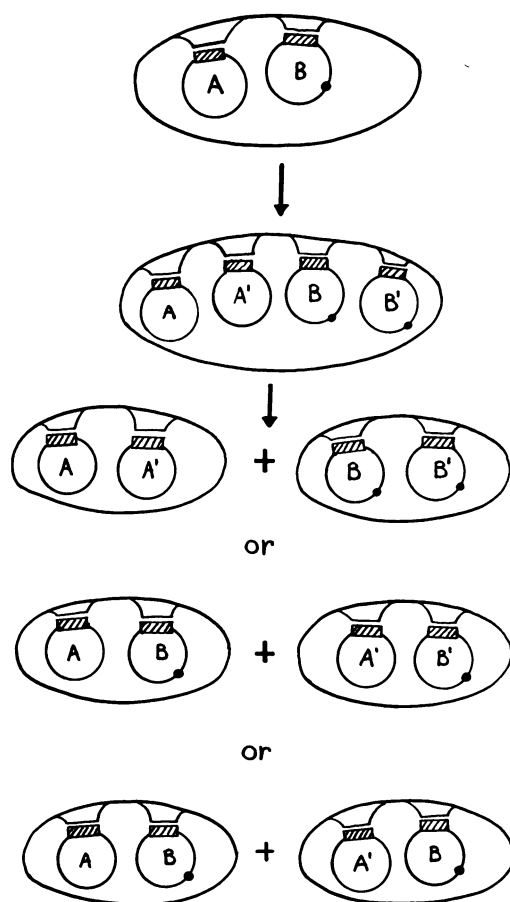


FIG. 5. Random segregation possibilities in a  $2 \rightleftharpoons 4$  cycle. One plasmid, B, is shown having a single allele different from the other, A, to illustrate heterozygosity or homozygosity of the daughter cells with respect to that allele. Note that there is only a one-third probability that any daughter cell will be homozygous.

always sent to each daughter cell, the two would be compatible. Careful physical studies might be sufficient to permit a differentiation between  $1 \rightleftharpoons 2$  cycles and higher multiples on the basis of the amount of plasmid DNA present.

(iii) A  $1 \rightleftharpoons 2$  cycle exists but is sufficiently irregular to allow incompatible heterozygotes to persist for a limited amount of time.

(iv) A  $1 \rightleftharpoons 2$  cycle exists but attachment is not required for replication, only for regular distribution of replicas. Recent studies in our laboratory suggest that this may indeed be the case for staphylococcal plasmids (R. Novick, unpublished data).

**Alternatives and modifications.** At least three supplementary possibilities deserve serious consideration. The first is a modification in which the

maintenance site is not present in the plasmid-negative cell but is induced there by some specific product of the entering plasmid. This modification would solve the possibly artificial problems of how the cell manages to produce precisely one maintenance site per generation for each of a large number of incompatibility sets and how a plasmid, entering, finds the right one. The induction of a site and its occupation by a plasmid must involve function of a plasmid gene (i.e., protein synthesis). Attachment must result in either a general perturbation of the cell membrane or the production of a repressor in order to prevent the future stabilization of an incoming plasmid of the same incompatibility set. This induction model fails to account for incompatibility set-specific host mutations affecting plasmid maintenance. As it stands, it also fails to provide a mechanism for segregation of plasmid replicas.

A different mechanism for incompatibility involves the production by a resident plasmid of a cytoplasmic, incompatibility set-specific repressor of plasmid replication. However, the action of a repressor cannot by itself account for known plasmid behavior; if plasmid segregation is nonrandom, then a structural component of the host cell must be involved. Therefore, the only repressor model worthy of consideration is one in which attachment to a maintenance site is responsible for segregation, and a repressor perhaps controls replication. (If attachment is also required for replication, then repressors become superfluous.) Pritchard (Heredity, *in press*), arguing for repressive control of replication, has proposed that a burst of repressor synthesis occurs once per generation, at the time replication is initiated. The repressor is diluted to a critical level during the next generation, whereupon a new round of replication ensues. In this case, one is faced with the necessity of an all-or-none response dependent on a twofold concentration change. Other plausible but complicated variations of the repressor model can be imagined but have little heuristic value and still less evidence and so will not be discussed here.

A repressor of F replication produced by integrated F would be most helpful toward an understanding of the Hfr/F incompatibility, a situation for which the maintenance site model offers no really satisfying explanation. However, the strongest evidence against the existence of a repressor is that, in the Hfr/F-*lac* system, it has been impossible to find a mutant Hfr that lacks it, despite the opportunity for strong selection in favor of such a mutant, namely selection for F-*lac* transfer to a *recA*, Lac<sup>-</sup> Hfr strain. In fact, the most that a diligent search has been able to

produce (W. Maas and A. Goldschmidt, Proc. Nat. Acad. Sci. U.S.A., *in press*) is an Hfr strain that accepts autonomous F-*lac* but carries an integrated F so defective that it can neither transfer nor replicate on its own and seems, therefore, to have suffered a gross deletion, including its determinant of incompatibility specificity.

Other evidence against "pure" repressor models is the observation that, in the staphylococcal system, replication of a superinfecting incompatible plasmid is arrested for some time, whereas the resident plasmid evidently continues to replicate (R. Novick, *unpublished data*).

A third model, suggested to me by Hirota, involves competition among incompatible plasmids for a cytoplasmic substance required for replication and produced by the host but in very short supply. This model by itself, like the repressor model, fails to account for nonrandom segregation and therefore requires the cooperation of a structural cell component. Its advantage is that, unlike the repressor model, it offers the incoming incompatible plasmid a fair chance of getting established. Also, it can account for persistent heterozygosity between incompatible plasmids and for incompatibility set-specific host mutants.

### Summary

A specific cell surface barrier that prevents contact formation or DNA transfer (or both) is responsible for the depressed fertility of crosses between two strains that both harbor the same sex factor. This barrier, whose chemical nature is unknown, is determined by the sex factor but does not involve sex pili. It is bypassed when the entering plasmid is injected by a phage particle (transduced).

Once past the entry barrier, the incoming plasmid must contend with another obstacle imposed by the resident one, namely, the occupation of a host maintenance system by the resident plasmid.

Cells provide specific maintenance systems or sites for plasmids. It is thought that attachment to such sites is required for replication and for segregation of replicas. Each plasmid is matched to a particular maintenance site through the specificity of part of its genome, its *mcr* region. A host cell provides for the regular inheritance of only a single plasmid matched to a particular maintenance site. Thus, different plasmids with isogenic or closely homologous *mcr* regions cannot be stably comaintained, i.e., are incompatible. Conversely, plasmids with different *mcr* regions can be stably comaintained, i.e., are compatible. Plasmids with the same maintenance site specificity form an incompatibility set. A plasmid

entering a cell already inhabited by another belonging to the same incompatibility set seems to reside unreplicated in the cytoplasm, sometimes for many cell generations, until by chance a free site becomes available.

Incompatibility is exhibited by all plasmids that have been tested; although details may vary, the basic mechanism seems universal.

### Integration

The now classic model for integration is provided by coliphage lambda, which integrates by a single crossover between circular phage and chromosome (31). This process has at least two requirements: a region of homology between episome and chromosome, and an enzyme catalyzing the exchange. The integration of  $\lambda$  has been excellently reviewed by Signer (241) and will be referred to here only insofar as it has relevance to the integration of sex factors and other plasmids. A simplified version is shown in Fig. 6

**Integration of plasmids.** The only plasmids aside from temperate phages that integrate with detectable frequency are sex factors belonging to the F incompatibility set, namely F and the ColV factors; it seems likely that the host recombination system is required for such integration, since Hfr formation is extremely rare in *recA* strains (W. Maas, *personal communication*). Hfr factors could have originated in such strains by a mechanism similar to that by which other non-*recA*-dependent events such as deletions occur (74), or they could reflect a specific but very inefficient F-linked integration system.

Like other recombinational events, integration crossovers could involve base-sequence homology or enzymatic recognition of specific, nonhomologous regions. In either case, specific pairing of matched sequences must occur. In the discussion that follows as well as in the section on recombination, I have made the assumption, for the sake of simplicity, that base sequence homology is required for crossing over. The discussion of integration and recombination would be equally well served by the alternative possibility of non-homologous but specifically matched crossover regions.

If homology between episome and chromosome is necessary for integration, it could be based on common ancestry for the regions involved or upon convergent evolution of base sequences. Alternatively, such homology could be fortuitous, involving base sequences that are sufficiently similar on a random basis to permit crossing over. There seems little doubt that the homology, or matching, between lambda and the *E. coli* chromosome

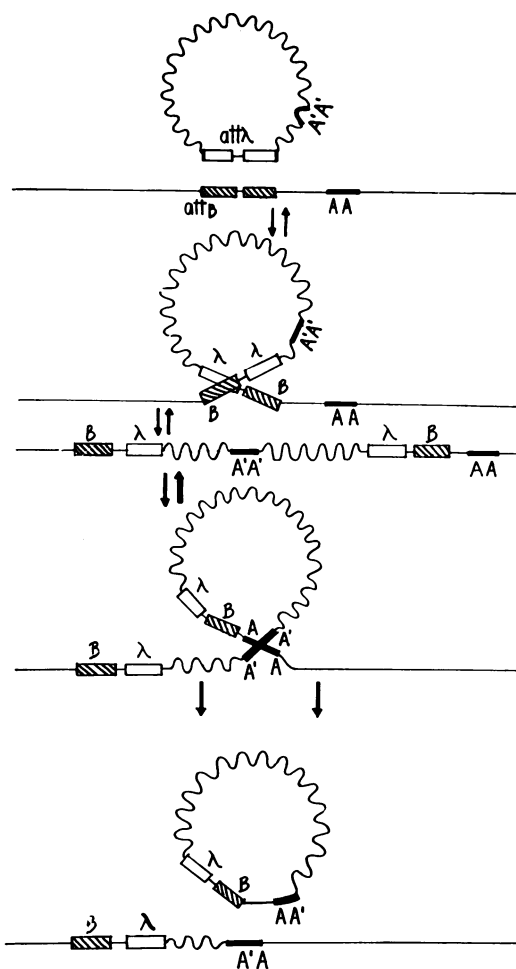


FIG. 6. Campbell model of episome integration and excision as illustrated by coliphage lambda. The upper sequence shows normal integration and its reverse involving homology between bacterial (*attB*) and phage (*attλ*) attachment sites to effect the single reciprocal exchange required. The lower sequence shows aberrant excision involving fortuitous or imperfect homology between regions *AA* and *A'A'* of the phage and chromosome, respectively, for the required crossover. The resulting phage genome has incorporated a segment of bacterial chromosome and is therefore a transducing phage.

is more than fortuitous, and the same is probably true for F (69). Since there are at least 13 different F integration sites (27), there must be many separate small regions, homologous or matching, scattered around the chromosome. If so, these regions could each be homologous to a separate region of F or could all be homologous to a single region. With an episome that has acquired a

region of host chromosome, that region overwhelmingly dictates activities dependent on homologous pairing. Since recombination between such merogenotes and chromosome is quite frequent, whereas recombination between F itself and chromosome is quite rare, the presumed genetic homology between F and chromosome is evidently so poor as to rule out long regions of recent common ancestry. Thus, events leading to F merogenote formation must have been unusual before F fell into the hands of Jacob and Adelberg (128). An apparently contradictory finding, namely that almost 50% of the DNA of F shows *base sequence* homology with the *E. coli* chromosome (69), can be interpreted as meaning that this homology must not be close enough to permit efficient crossing over. Since the known plasmid hosts have perfectly good recombination enzymes, most plasmids cannot have gross regions of genetic homology with their host chromosome, because they are unable to integrate. Such plasmids may, of course, possess specific integration mechanisms of their own but fail to integrate because they currently occupy hosts lacking the requisite homology regions.

**Integration of plasmid fragments.** Aside from integration of intact episomes and the formation of F merogenotes and transducing phages, there are several examples of genetic exchange between chromosomes and plasmids in either direction.

Nontransmissible but stably inherited fragments of R factors are produced spontaneously and during conjugal or transductional transfer (see review by Watanabe, 270). It has often been assumed, without strong evidence, that these fragments are integrated into the chromosome and that this integration is due to plasmid-chromosome homology. An example of chromosomal integration of R factor-linked tetracycline and streptomycin resistance has been documented by Dubnau and Stocker (61). Strains carrying integrated resistance loci were isolated as P22 transductants unable to transmit their R genes conjugally. However, the transductants were P22 immune and defective, the R genes were integrated at the P22 integration site, and one such transductant produced phage lysates transducing tetracycline resistance at high frequency. The authors concluded that integration was by virtue of attachment to the phage genome rather than by virtue of any intrinsic homology between R factor and chromosome. Other reports of integration (100) have failed to take account of the possibility that defective phages were involved. Penicillinase plasmid fragments carrying an erythromycin resistance marker (192) or a penicillinase

marker (L. Wyman, *personal communication*) were rarely observed to integrate into the host chromosome at unlinked sites. Evidence for involvement of defective or cryptic prophages could not be found in either case, so that these examples may represent integration of plasmid fragments alone.

The rarity of these events suggests that, if crossing over is involved, it is a sporadic event based on fortuitous homology and does not necessarily speak for ancestral relationships between the genomes involved or for any plasmid-specific integration-excision mechanism.

**Mutations consequent to integration and excision.** Episome-chromosome interactions leading to altered host function have been observed with certain phages, with F, and with silent DNA fragments (239). These interactions have always resulted in stable mutational alterations of the chromosome as a consequence of episome integration or excision (or both). An example is bacteriophage *Mul* (261), which can integrate at many sites along the *E. coli* chromosome, producing mutations that are evidently irreversible.

A second well-studied example involves F (14, 27, 45, 235), which occasionally integrates within the confines of a known cistron, splitting it into two sections; these are transferred separately during conjugation as origin and terminus, respectively, of the resulting Hfr chromosome. Release of integrated F factors is not always an exact reversal of the integration event. Sometimes a segment of chromosome is incorporated into the episome, forming an F merogenote, and a segment of the sex factor is left behind as a sex factor affinity site (219). Again, the result is a deletion (230). Host strains derived from such events act as intermediate frequency donors of markers near this site when infected with a wild-type F. This finding is further evidence that wild-type F does not have any sizeable regions of recent common ancestry with the chromosome; if it did, it would transfer at high frequency markers near such regions.

Several strongly polar stable point mutations in the *E. coli gal* operon were found by Shapiro (239) to be owing to the insertion of a silent stretch of DNA at each of the respective mutant sites. The extra DNA was reflected in an increased buoyant density of  $\lambda dg$  particles carrying the mutant loci; *gal*<sup>+</sup> back mutations returned the  $\lambda dg$  density to its original value. This is the only known example of reversible insertion mutations.

Insertion and excision of prophages are not ordinarily mutagenic—even though  $\lambda$  can occasionally incorporate chromosomal regions adjacent to its attachment site; strains cured of  $\lambda$  do

not normally suffer deletions of these regions. [See review by Scaife (230) for a discussion of integration and excision.] P2, however, often undergoes anomalous excision in producing spontaneous cures. One of the P2 prophage sites is near the histidine region in *E. coli* K-12; lysogens at this site frequently segregate *his* mutants which turn out to be cured of P2 and to have deletions involving the histidine region (143). Thus, mutations consequent to episome integration are stable and are usually irreversible. No unstable mutations or other plasmid-chromosome interactions have been observed that might be due to the effects of "controlling episomes" postulated by Dawson and Smith-Keary (53), nor have specific "mutator" episomes acting on specific chromosomal loci been seen. Two examples of elevated mutation frequency of specific loci thought to represent "mutator episome" effects both involved transmissible R factors (88, 94, 95) and can be interpreted as the occurrence of mutations whose effects are synergistic to R-linked resistance determinants, and whose occurrence is facilitated by residual growth of the R<sup>+</sup> cells in the presence of the drug at a selective concentration (208).

#### Formation of Merogenotes

"Classical" F merogenotes (128) can be isolated in Hfr × F<sup>-</sup> crosses by selection for a late marker after early interruption of mating. They are formed by a rare excision crossover involving paired chromosome regions on either side of the integrated sex factor or by one involving a chromosomal site and an homologous F region [see Scaife (230) for diagrams and discussion of this mechanism.] Thus, they are already present in donor cells before the start of mating. Presumably because of the location of excision crossovers, "classical" merogenotes generally include all or most of the F genome.

Recently, Low (159) described a new procedure for obtaining F merogenotes that promises to provide coverage by these elements of the entire *E. coli* map. Most of these new F merogenotes are defective and their study should reveal a great deal about the organization and integration of F as well as about the mobilization process. In crosses between Hfr donors and F<sup>-</sup> *recA* recipients at low frequency, Low found "recombinants" that were invariably heterozygous for proximal markers, including those nearest to the Hfr origin. This heterozygosity was attributable to F merogenotes, some of which were fertility-defective. Many Hfr donors were tested; some produced no detectable "recombinants," some produced exclusively defective F merogenotes, and others

produced mostly but not exclusively normal ones. Many of the Hfr donors gave rise to several classes of merogenotes with regard to the chromosomal markers included, but 10 merogenotes out of 11 examined carried only the Hfr origin plus proximal markers. Only one carried markers from the chromosome terminus. These 11 were derived from 6 different Hfr donors.

I would like to suggest that many of the merogenotes carrying only proximal markers were formed by a mechanism different from that usually associated with F' factor formation, namely, within the *recA* recipient after mating, by cyclization of a proximal segment of the chromosome, including the origin and that part of the F linked to it (159). This possibility gave rise to the following model.

(i) The F factor has a number of separate regions, each having a degree of homology with a different chromosomal site (rather than one F region homologous to each of the several chromosomal sites).

(ii) As a consequence of integration by recombination involving different homologous pairing regions, the integrated F factors in different Hfr strains will form a series of circular permutations of the F genome, for example, as in Fig. 7.

(iii) The transfer origin corresponds to a specific local region of the F genome, where the chromosome is always opened during conjugation. In the rolling circle model for replication (Fig. 16; see "Replication"), this region would correspond to the site of action of a specific nuclease.

(iv) The proximal chromosome region transferred by a particular Hfr will then include only that part of the F genome which happens to follow the origin in the particular F permutation present.

(v) Those few fragments which have ends appropriate for cyclization would then form circles. (Cohesive ends would presumably have to be involved, since recombination between homologous duplex ends is unlikely in the *recA* recipient.) Only those Hfr donors in which the F replicator region was on the proximal side of the origin could form autonomous merogenotes. Those that had the fertility region as well would act as F' donors and those that had the *tau* region would confer resistance to the female-specific phage tau. Since all the F' strains were resistant to tau, linkage of *tau* to the *rep* region is suggested.

If linear DNA is transferred, the generation of cohesive ends poses a problem that weakens this argument but is not insoluble. Because different homology requirements involving the sex factor and the nearby chromosome must exist for the

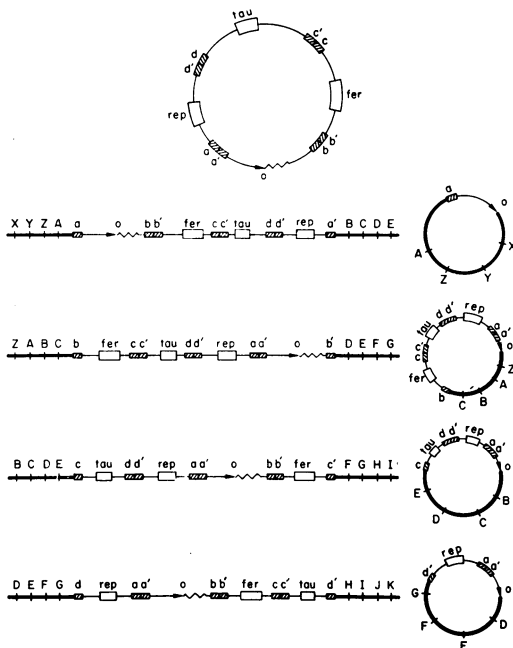


FIG. 7. *F* integration and proximal *F* merogenote formation in *Hfr recA*  $\times$  *F<sup>-</sup> recA* crosses. The diagram shows a map of *F* with an arbitrary arrangement of *F* regions concerned with replication (*rep*), fertility (*fer*), and resistance to phage  $\tau$  and the transfer origin (*o*). The four different circular permutations result from integration crossovers at four sites (*a*, *b*, *c*, and *d*), each homologous to a different chromosomal region. The smaller circles represent four classes of proximal merogenotes, one corresponding to each *F* permutation, that can arise in matings of *Hfr* donors with *recA* recipients. The first of these would not be able to replicate autonomously; the other three would. Only the second would be able to act as a sex factor. Host markers are represented by capital letters.

formation of classical (recombination) and proximal (cyclization) merogenotes, one expects that with *recA* recipients some *Hfr* strains would give rise to neither type of merogenote, some would give rise to one type, and others to both (159). In a *recA*  $\times$  *recA* cross, however, one would expect either proximal merogenotes or none. Whereas various *Hfr* strains should differ with respect to the inclusion of *F* genes in the proximal merogenote to which they give rise, any particular *Hfr* should give rise to but a single class (Fig. 7). Thus, analysis of the proximal merogenotes produced by several *Hfr* donors should permit the construction of a permutation map of *F* including its various integration-crossover sites.

#### Formation of Hft Elements

Hft elements are composed of bacterial genes linked permanently to intact or defective phage

genomes. There are at least two different mechanisms involved in their formation: that of aberrant excision of integrated prophages (31; Fig. 6), and that of rare direct recombination between phages and bacterial genes that are ordinarily unlinked. Such Hft derivatives of a number of generalized transducing phages [P22 (61), P1 (161),  $\epsilon$  (141), and P11 (194)] have been isolated. The bacterial genes most commonly involved are derived from extrachromosomal elements, a fact which may reflect a more direct evolutionary relationship between generalized transducing phages and extrachromosomal elements than between these phages and the chromosomes of their host bacteria. In no case is it known whether homologous crossover was involved. Since the recombination events that have given rise to Hft derivatives of generalized transducing phages are rare and sporadic, they are not readily subject to experimental scrutiny. With P1, however, the formation of Hft P1*dl* elements can be induced under controlled conditions (161), and their genetic analysis in terms of the relationship between the *lac* genes and the P1 genome may be explored in detail.

In some cases, the phage moiety of an Hft element is nondefective. Examples are  $\lambda$ *pb* (282), P1*pchl* (146), and  $\phi$ 80*pt* (168). In such cases, the only distinction between an Hft element and a converting phage is that the former carries known chromosomal genes, whereas the latter carries somatic determinants of unknown origin.

#### Mechanism of Genetic Transfer

Chromosomal mobilization by *F* merogenotes through recombination is well understood and has been discussed thoroughly by Scaife (230). However, the mechanics of the process by which sex factors bring about transfer of genetic material, even their own, remains obscure. Sex factors evidently direct the production of conjugation tubes thought to be located at the hypothetical membrane attachment site for the sex factor (130). This may be true, but an additional factor would seem to be necessary to direct a DNA molecule into the tube. It is possible that mutants of the sex factor which abolish conjugal fertility without detectably affecting the formation of sex pili (203) could involve such a hypothetical "transfer factor."

Genetic elements other than sex factors are conjugally transferred. Since nontransmissible plasmids are usually mobilized much more frequently than chromosomal fragments, it has been suggested that transient "association" (i.e., linkage by associative recombination) between such plasmids and the sex factor is responsible (6). In view of the finding that plasmid cotransfer frequencies are the same with *rec<sup>+</sup>* and *recA* donors (R. Clowes, *personal communication*), it seems

unlikely that such association could be a significant factor, unless plasmids have their own very efficient recombination systems. Alternatives are that cotransfer is purely fortuitous or that transmissible plasmids direct the production of a factor which is required specifically for the initiation of transfer and which acts on other genetic elements as well as on the sex factor itself. There are widely differing cotransfer frequencies for various combinations of elements; these variations could reflect differences in the efficacy of "transfer factor" produced by different sex factors for various nontransmissible plasmids.

Consistent with the idea of transfer specificity is the finding by Romero and Meynell (221) that an R(f) and an R(i) factor in the same cell are transferred to R<sup>-</sup> recipients independently of one another and do not seem able to share conjugation tubes. Thus, when one is fertility-derepressed and transfers at high frequency, the other continues to transfer at its original low frequency. This result seems contradictory to an earlier finding of Clowes (38) that CoI can restore the fertility of a defective Hfr.

Sex-factor-mediated transfer of chromosomal markers is largely dependent on the normal recombination system. This transfer may therefore involve transient linkage between sex factor and chromosome. *RecA* strains carrying various sex factors can all transfer large chromosome fragments at roughly the same low-residual frequency (about 10<sup>-8</sup>/donor cell) (39). For F this frequency represents a 30-fold reduction in efficiency in comparison with *rec*<sup>+</sup>; for other sex factors, notably CoI, this frequency is the same as with *rec*<sup>+</sup> donors. These differences evidently reflect differences in degree of homology between sex factor and chromosome that lead to widely varying frequencies of recombination-dependent transfer. With *recA* donors, Clowes and Moody (39) found frequent cotransfer of unselected markers 180° apart on the map; the distribution of unselected donor markers among the recombinants was similar for all of the sex factors. These findings suggest the existence of a general mechanism for recombination-independent transfer.

### Summary

Regions of good base sequence homology between naturally occurring plasmids and host chromosomes are rare. Recombination events leading to integration of intact plasmids or fragments or to the formation of merogenotes are exceptional and probably represent crossovers between regions of relatively poor homology. The same seems true of recombination between different plasmids or plasmids and phages.

Deletions and stable point mutations some-

times occur consequent to integration or excision (or both) of temperate phages, episomic plasmids, and other DNA fragments. Stable and unstable mutations consequent to attack by known plasmids on specific sites have not been observed.

Genetic transfer by sex factors is largely dependent upon normal recombination mechanisms and may involve transient linkage. Transfer occurring independently of normal recombination mechanisms is rare and may involve the operation of a sex-factor-specific "transfer substance" which is required for transfer of the sex factor itself.

## GENETIC ANALYSIS

### General Considerations

It seems probable that plasmid essential functions comprise the basic minimum genetic structure required for controlled autonomous replication. If it should be true that these essential functions obligately occupy an exclusive localized region of a plasmid, then detailed genetic analysis of this region should reveal the organization and content of the minimal self-replicating unit. This analysis must take into account three basic properties of plasmids: replicative autonomy, circularity, and most maintenance site requirements. Whereas the fact that plasmids are small autonomous units has facilitated genetic studies, host maintenance site specificity has so far greatly complicated complementation and recombination analysis. Although, in consequence, it has not thus far been possible to obtain a complete linkage map based on recombination for any plasmid, deletion studies have been more fruitful and have provided a framework within which to evaluate recombination and complementation data. As will be seen, recombination experiments so far have been more revealing of basic events in the plasmid life cycle than of linkage relationships between markers.

### Deletion Analysis

Most plasmids are transferred intact by conjugation or by transduction. Lacking good homology with the host chromosome, they do not lose alleles by recombining with it after transfer. However, plasmids do occasionally lose markers by deletion; this loss seems to occur more frequently during transfer than spontaneously (192, 273).

Deletion mapping assumes that the majority of deletions remove a single continuous segment; simultaneous deletion of two or more noncontiguous segments is assumed to be comparatively rare. Two simple mechanisms for deletion of continuous segments are shown in Fig. 8: excision-loop formation involving a single reciprocal cross-

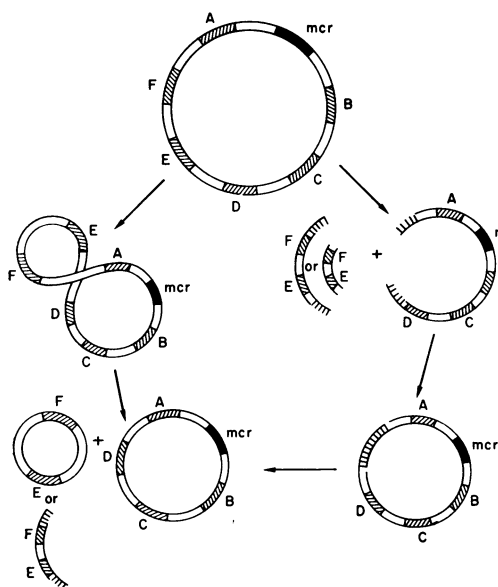


FIG. 8. Possible models for the formation of deleted plasmids. Various plasmid markers are represented by capital letters; *mcr* stands for the maintenance-compatibility-replication region of a penicillinase plasmid. Fragments lacking this region are nonviable. In the figure-eight model, if the crossover is reciprocal, a small nonviable circle is deleted. If the crossover is not reciprocal, the deleted fragment is linear. In the other model, a fragment is excised by two pairs of staggered single-strand scissions or by one pair of double strand cuts. In the former case, the two fragments have single-stranded ends. In the latter case, single-stranded ends could be generated enzymatically. In both cases, only those deleted plasmids would survive whose single-stranded ends were sufficiently homologous to be cohesive.

over (74), and two separate double-stranded breaks followed by rejoining of the free ends. Such rejoining could involve cohesive sites exposed by the action of an exonuclease; both mechanisms would then require regions of intramolecular homology. However, in view of the lack of requirement for any known recombination system in the production of deletions (74), it is questionable whether homology is necessary.

Because most plasmid genes are nonessential, deletion analysis is limited more by structural factors than by genetic function. Only those deletions will survive whose ends are compatible for recircularization. One expects to encounter regions that cannot be deleted without loss of plasmid autonomy; such regions will act as deletion barriers. Thus two important topographical features of the resulting map will be deletion "hot spots," which may reflect regions of intramolecu-

lar homology, and deletion barriers, which reflect the location of essential genes. Marker orders should emerge, but distance estimates based on deletion frequencies are likely to be grossly distorted. Deletion mapping may be complicated by the survival of nonviable fragments (Fig. 8) through integration into another functional replicon. However, such fragments should then differ recognizably from their plasmid-linked homologues. The possibility also exists that another replicon could supply in *trans* a diffusible essential substance whose corresponding determinant was lost by deletion. This determinant would not be recognizable as an essential function unless a host could be found that failed to supply it.

Several plasmids have been subjected to deletion analysis, including  $PI_{258}$  (192), *F-gal* (203), and a number of derivatives of ColV, B-K260 (P. Fredericq, Ciba Found. Symp., *in press*). Because all of these elements are known to be physically circular, the maps are so represented (Fig. 9-11). Deletions of  $PI_{258}$ , obtained among

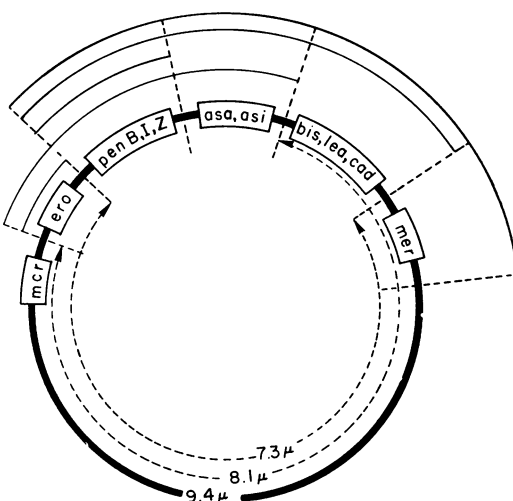


FIG. 9. Deletion map of  $PI_{258}$ . Deleted segments are represented by solid lines outside the map itself. See Table 1 for explanation of symbols. The termination points for deletions are arbitrary, e.g., there is no certainty that all deletions terminating between *pen* and *ero* do so at the same site. Numbers refer to contour lengths in microns of the parental plasmid and of two derivatives, each bearing a deletion whose genetic extent is indicated by the corresponding gap. The measurements were made by Charles Gordon on electron micrographs of plasmid DNA prepared by Mark Rush. Contour length measurements of  $\phi X174$  RF included in each preparation as a reference did not vary by more than  $\pm 3\%$  from preparation to preparation. Thus the plasmid lengths given are a good representation of their comparative sizes.

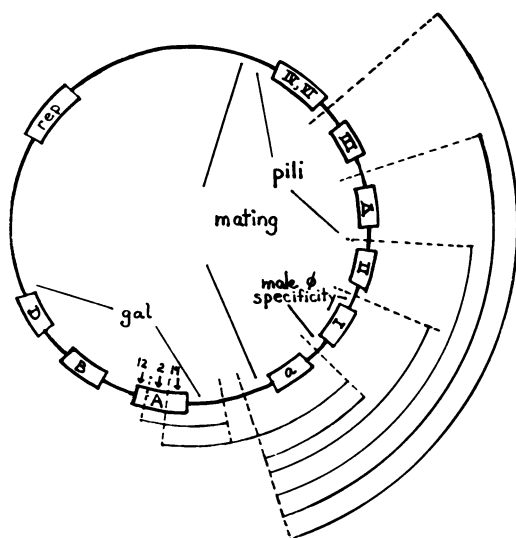


FIG. 10. Deletion map of F-gal. This map is represented as a circle on the basis of physical evidence for circularity of the corresponding DNA molecule (80). The data of Otsubo (203) which serve to orient the gal and fer regions with respect to one another say nothing about a replication region. The location of such a region, rep, is forced by Otsubo's isolation of a deletion that spans the interval between galA and the first fertility cistron, a. The resulting Gal<sup>-</sup>, Fer<sup>-</sup> plasmid was still autonomous so that the deletion could not have included any essential region. Other F functions that must for the same reason be located near rep are T3 restriction and resistance to phage  $\tau$ . Numbered sites in the galA cistron that are indicated by arrows served to locate the terminations of two deletions.

transductants by scoring for unselected plasmid markers, were rare unless the transducing lysate was UV-irradiated. This treatment increased their frequency to several per cent. PI<sub>258</sub> carries markers for resistance to penicillin, erythromycin, and several inorganic ions (192, 196), 11 known cistrons in all. Its deletion map is characterized by several "hot spots." Most of the deletions shown in Fig. 9 occurred many times. These "hot spots" are not reflected in physical distances. Whereas most deletions terminated among the known markers (192), contour length measurements of isolated DNA corresponding to the parental plasmid and two of the deletions (225) revealed that the known somatic determinants occupy not more than one-fourth of the plasmid molecule (Fig. 9). One barrier was encountered, that occupied by the *mcr* region. Among transductants selected for penicillin or for cadmium resistance, no deletions were found that extended counter-clockwise beyond this region. Among transductants selected for erythromycin resistance, most deletions terminated before the *mer*

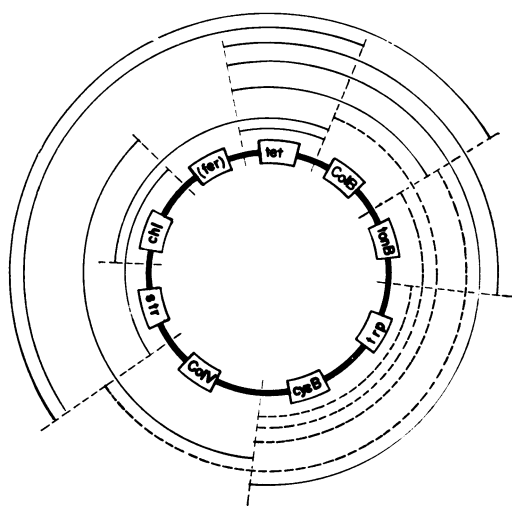


FIG. 11. Transduction-deletion map of a Col V, B derivative (P. Fredericq, personal communication). Markers belonging to the plasmid are indicated in the boxes (see Table I); arcs surrounding the map represent fragments transduced by phage P1. Dashed lines represent different fragments obtained by selection of transductants for tryptophan independence; solid lines represent results of selection for tetracycline or chloramphenicol resistance. The *fer* locus could not be placed unambiguously on this map.

locus. However, a class extending clockwise beyond this locus was encountered. Seventeen of 18 deletions in this class included the *mcr* region, leaving the *ero* locus as the only recognizable plasmid marker. These *ero* fragments, however, were no longer autonomous and appeared to be integrated into the host chromosome. The residual fragments representing all other classes of deletions remained autonomous as shown by compatibility tests. Thus it could be concluded that a region between *ero* and *mer* was both necessary and sufficient for autonomous replication (192).

Deletions of R factors, both spontaneous and during transfer, have been observed and documented (270) but have not lent themselves to the production of a map. Deletion frequencies have been helpful in establishing linkages that were confirmed by recombination analysis (102) as discussed below.

An F merogenote with a built-in chromosomal segment of known gene sequence was utilized to good effect by Otsubo (203) in his studies of the genetic determination of fertility. F-gal was transduced by phage P1 to a *recA*galB recipient with selection for Gal<sup>+</sup>. Some of the transduced merogenotes were found to have deletions of the F-linked *galA* locus and to be fertility-defective as

well. These deletions must have spanned the region between the F-linked *gal* and fertility determinants, showing that the two are linked. Since *galA* but not *galB* was deleted, the *gal* operon must be oriented with respect to the fertility region as shown in Fig. 10. Other deletions involving only the fertility region were also found. These were mapped by complementation with fertility-defective point mutants of an R(f) factor that had previously been grouped into cistrons on the basis of phenotype and of complementation behavior with fertility-defective F-*gal* mutants (see "Fertility"). Examination of these data established the orientation of the fertility genes with respect to the *gal* operon (203). These results must be considered preliminary; localizations of the fertility-defective F-linked and R-linked point mutations that were used to determine the extent of the deletions are not absolutely certain (203). A glance at this map places two of the remaining known F-linked determinants, autonomous replication (*rep*) and resistance to phage tau, beyond the fertility cluster in a counterclockwise direction, since a deletion spanning the interval from *galB* to *fer* did not affect them.

Fredericq (Ciba Found. Symp., *in press*) has constructed, by recombination, a series of complex sex factors carrying genes derived from ColV, B-K260, R(f), F-*lac*, and the *cys-trp* region of the *E. coli* chromosome. DNA corresponding to one of these, ColV, *cys trp*, has been isolated (110) and contour length measurements have assigned to it a molecular weight of  $1.07 \times 10^8$  (Table 5). The others (Fig. 11) have additional markers and may be larger. Phage P1 cannot ordinarily transduce DNA molecules of this size; transduction by P1 produces a series of plasmid deletions. Figure 10 shows the types of different plasmid fragments transduced. By piecing such fragments together, Fredericq was able to construct circular deletion maps for the plasmid represented in Fig. 11 as well as for several others (Ciba Found. Symp., *in press*). The analysis of these transduced fragments has not yet been carried to the point of determining which ones are autonomous and, therefore, the location of essential genes is still unknown.

### Recombination Analysis

Plasmids undergo recombination presumably by molecular mechanisms similar to those for other homologous genetic elements. Plasmid recombination analysis is, however, complicated by persistent heterozygosity and by maintenance site requirements and, consequently, is still relatively rudimentary. In the following discussion, plasmids are depicted as circular in accordance

with physical data. "Map" circularity has been inferred from deletion data for the ColV, B derivative illustrated in Fig. 11 and from a combination of deletion and recombination data for the staphylococcal plasmid shown in Fig. 9.

**Model for recombination between isogenic, incompatible plasmids.** Figure 12 presents a simple model for recombination between incompatible plasmids that is based partly on hypothetical and partly on experimental considerations. This scheme shows the usual situation in plasmid crosses—introduction of one plasmid (the super-infecting one) into a cell harboring another (the resident one)—and it has the following features (numbered to correspond with the numbered steps in Fig. 12). Crossovers are assumed to be reciprocal.

I. The incoming plasmid enters as linear DNA molecule. This is known to be the case for conjugation (41, 166) and seems probable but has not been proven for transduction. If it is single-stranded, a complementary strand is rapidly synthesized (41).

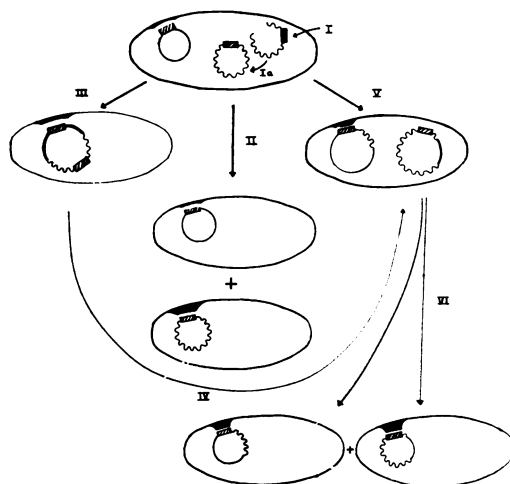


FIG. 12. Possible events in recombination between incompatible plasmids. Though isogenic, the two are represented differently for identification. A maintenance site is represented by a thickened area of the cell wall; the corresponding plasmid region is shown as a cross-hatched box. Numbered stages are: I, superinfection; Ia, circularization; II, segregation without recombination; III, associative recombination; IV, dissociative recombination producing a pair of reciprocally recombinant plasmids; V, commutative recombination combining III and IV in a single step; VI, segregation of recombinant plasmids. Note that segregation in stages II and IV produces reciprocally homozygous progeny.

Ia. It then circularizes; crossing over is theoretically possible either before or after cyclization. If recombination occurs before cyclization and the free ends later join, the net result is the same as if cyclization occurred initially. Conceivably, a newly recombinant linear plasmid could have different properties than a newly recombinant circular one.

II. The two plasmids may segregate without recombining.

III. An odd number of crossovers may occur, producing a heterozygous double plasmid ("associative" recombination; 227). Being a tandem duplication, a heterozygous double is inherently unstable in the presence of a functional recombination system. As double plasmids formed by associative recombination involve fusion of two independent replicons, they should have special properties resulting from the presence of two operative replication systems.

IV. The double undergoes an odd number of internal crossovers, producing a pair of singles ("dissociative" recombination). Since the double has homologous halves, a dissociative crossover is unlikely to occur at the same site as the associative event by which the double was formed; thus, the products are likely to be recombinant. If a nonhomologous dissociative crossover occurs, two unequal circles will result, one of which has a deletion, the other a duplication of part of the plasmid. This possibility is mentioned because deletions and partially heterozygous recombinants have been observed in plasmid crosses (102, 190; R. Novick, *unpublished data*).

V. An even number of crossovers occurs initially, producing a pair of recombinant single plasmids ("commutative" recombination).

It could be argued that, if a double plasmid were to segregate rapidly, there would be no difference between paired associative-dissociative events and a single commutative event. Any functional difference between the two thus hinges on whether the double has a finite existence in time and the dissociative crossover requires a separate homologous pairing event.

In this model, the production of single-length recombinants requires ultimately an even number of crossovers. Recombination maps derived from a consideration of such recombinants should then be circular, unless there is an obligatory location for one of the crossovers, in which case they would be linear (253). Recombination studies with staphylococcal plasmids and with R factors have failed so far to yield data that effectively test this scheme. A consideration of these results is instructive because they have brought to light other factors that bear on plasmid recombination.

**Results with staphylococcal plasmids.** In this section, results quoted without references are from unpublished experiments by the author.

(i) Independent  $PI_{258}$ -linked mutations to cadmium, arsenate, and erythromycin sensitivity (196) and others involving penicillinase structure or inducibility (189) have been utilized as marker alleles in plasmid crosses.

In transductional crosses, a resident plasmid incompatible with the entering one does not depress the frequency of transduction of the latter. In 80 to 90% of transductant clones selected for one donor plasmid marker, the donor plasmid has replaced the resident one: all donor alleles are present and none of the original resident ones are detectable; the other 10 to 20% harbor homozygous recombinant plasmids; rarely do heterozygous clones occur.

(ii) Joint selection for a donor and a recipient marker in different cistrons reduces the transduction frequency by a factor of 10 to 100, and all of the transductants are unstably heterozygous for all of the markers in the cross. Such heterozygous clones segregate parental plasmid types and a variety of recombinants; linkage and gene frequency analysis of the segregants has been unrewarding.

(iii) The problem of persistent heterozygosity can be eliminated by selection for intracistronic recombination. Scoring of three unselected plasmid markers in several crosses between non-identical cadmium-sensitive mutants revealed a very clear pattern. In pairs of reciprocal crosses, all of the unselected markers were contributed by the recipient plasmid in 90 to 95% of recombinants. Similar results were obtained when transductants selected only for a donor marker were examined. Among those that were recombinant for any one recipient marker, at least 95% received all of the other recipient markers as well. One conclusion from these results is that recombinants are formed by an even number of crossovers within a region of effective pairing that is very short by comparison with distances between loci.

This situation (Fig. 13) will make it impossible to obtain a linkage map by recombination, unless closely linked markers can be examined. An even number of crossovers within a short pairing region should produce two classes of cadmium resistant recombinants: recipient-like and donor-like (Fig. 13). As noted, the results show a strong bias in favor of the former; any full interpretation must account for this bias in the face of the ability of nonrecombinant donor plasmids to establish in the same recipient.

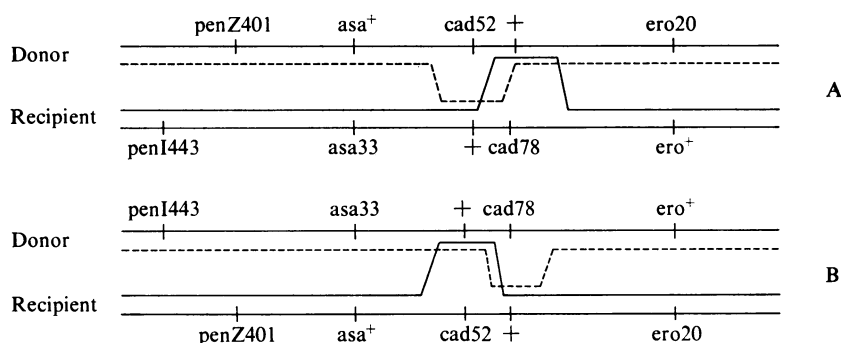


FIG. 13. Plasmid cross illustrating paired crossovers in a limited region of synapsis and recipient bias. Solid lines indicate recipient-like genotypes of predominant *CadR* recombinants. Dashed lines indicate rare donor-like recombinants. A and B are reciprocal crosses. Maps are represented as linear for clarity.

The recipient bias among recombinant plasmids must be a reflection of the fact that, as noted, the entering plasmid is on a different footing from the resident one. The bias can be understood either as the failure of donor-like recombinants to form, or as their destruction once formed. Three possibilities may be considered.

(i) Although most plasmid transductants receive the entire donor plasmid, those destined to form recombinants receive only small fragments.

(ii) There are several copies of the resident plasmid, crossing over is promiscuous, and the frequency of unselected donor alleles among recombinants is simply a reflection of the original ratio of donor to recipient in the zygote (102, 257).

(iii) Donor-like and recipient-like recombinants are formed in equal numbers, but the former are subsequently lost or destroyed, despite the fact that donor-type nonrecombinant plasmids survive perfectly well in the same cross.

The first possibility seems unlikely, because transducing particles destined to produce recombinants are inactivated by UV at nearly the same rate as those that are destined to transduce an intact donor plasmid (L. Wexler and R. Novick, *unpublished data*); if the former represented only small fragments, they should be very much less sensitive to UV.

The second possibility is unlikely for two reasons. First, between 10 and 20 copies of the resident plasmid would be required and other evidence suggests that not more than one or two are present (*see* "Basis of Incompatibility"); second, the promiscuous recombination required would have to involve only newly recombinant donor plasmids but not nonrecombinant ones, since between 80 and 90% of entering plasmids eventually segregate without visibly recombining.

The third possibility seems intuitively unlikely, but it is the only one supported by any evidence.

The pattern of unselected markers emerging from a cross was drastically changed by the introduction of a plasmid-linked *seg*<sup>-</sup> allele into the crosses. This allele results in exponential dilution of the plasmid during growth of its host strain at 42 C but not at 32 C (193) and is analogous to the *F<sub>1</sub>-lac* mutants described by Jacob et al. (130). When the recipient plasmid carried the *seg*<sup>-</sup> allele in crosses carried out at 32 C, the overwhelming majority of recombinants had donor alleles for the unselected markers instead of recipient alleles, as was seen with *seg*<sup>+</sup>. (The *seg*<sup>-</sup> plasmid is not entirely normal at 32 C.) In the reciprocal situation, with the *seg*<sup>-</sup> plasmid as donor, the result was an even greater bias toward recipient alleles. After selection for a single donor marker, no donor-type transductants were observed—all were recombinant and, among the donor markers, only the one selected was recovered.

These observations are inconsistent with the hypotheses that the recipient bias is due to fragmentation or to multiple copies in the recipient. The following conclusions are suggested.

(i) Unlike intact superinfecting plasmids, recombinant plasmids must attach to a host maintenance site when formed or else they are lost (destroyed?). The molecular difference between recombinant and nonrecombinant superinfecting plasmids is unknown.

(ii) The *seg*<sup>-</sup> mutation drastically alters the ability of the affected plasmid to compete for its maintenance site. The superinfecting normal plasmid evidently preempts the site, displacing the *seg*<sup>-</sup> or preventing it from becoming established even at low temperatures.

(iii) The *seg*<sup>-</sup> defect is a point mutation (reversions to temperature stability have been demonstrated), and its segregation behavior suggests that it is unable to replicate at 42 C. Thus, a mutation

evidently affecting replication adversely affects the ability of the plasmid to compete for a host maintenance site.

**Compatible staphylococcal plasmids.** A possible advantage of compatible plasmids in mapping studies is that one can begin with a stable heterodiploid on which both plasmids are on an equal footing, and there is no reason to worry about fragments or numerical asymmetry (Fig. 14). A possible disadvantage is that homology between the two *mcr* regions may be so poor as to preclude recombination or complementation (or both) among *mcr* cistrons.

Richmond (216) has presented evidence, based on cotransduction, for relatively stable heterozygous double plasmids. However, it is not clear whether long-lived associative recombinants are obligatory recombination intermediates. The net effect of paired associative-dissociative or of commutative events is a pair of recombinant plasmids that are still compatible. If a cross involves only markers in different cistrons, no change in host phenotype results. Detection and analysis of such recombinants thus requires trans-

ductional outcrosses of heterodiploid clones (192; R. Novick, *unpublished data*). Analysis of this type has revealed that when a diploid harbors recombinant plasmids, they are usually reciprocal. Hence, the term "commutative" is used in reference to the schemes in Fig. 12 and 14. Further, there is no particular bias among recombinants toward markers derived from the original resident or superinfecting plasmid in the diploid. Analysis of recombination within plasmid diploids, however, has not yet progressed to the point where anything can be said about linkage, limited pairing regions, or circularity.

**R factors.** Recombination studies with incompatible R factors (102, 104) have produced results roughly similar to those with staphylococcal plasmids. A strong recipient bias has been found both for conjugational and for transductional crosses, and linkage has been difficult to demonstrate. All meaningful crosses have been done with selection for intracistronic recombinants because persistent heterozygosity precludes the selection of interacistronic crossovers. Two-factor crosses between pairs of *tet* mutants and pairs of *chl* mutants have revealed the order of several sites in the *tet* locus with respect to each other and to *fer* and of several sites in the *chl* locus with respect to *str*, but the recombination data do not permit the construction of a consistent linkage map (102, 104). Transmissible R factors belonging to different compatibility sets were reported not to undergo recombination (276); however, intracistronic crossovers have not been selected, nor have transductional outcrosses for recombinants been done with R(f)/R(i) heterodiploids. Thus, it seems more likely that the failure is of observation rather than of occurrence.

#### Recombination between Heterogenic Plasmids

The assembly of composite plasmids carrying nonallelic markers from two or more heterogenic parental elements has been observed repeatedly. The best-known example is the formation of classical F merogenotes; other examples are the assembly of an R(f) factor carrying four resistance markers by recombination between one carrying three, *str*, *sul*, *chl*, and one carrying one, *tet* (270), and the formation of Hft elements. The farthest this process has been carried in the laboratory is the construction of a composite sex factor similar to the one mapped in Fig. 11, containing genes derived from F-*lac*, ColV, B, an R factor, and the chromosome (P. Fredericq, Ciba Found. Symp., *in press*).

The recombination events leading to the formation of such composite plasmids may be of evolu-

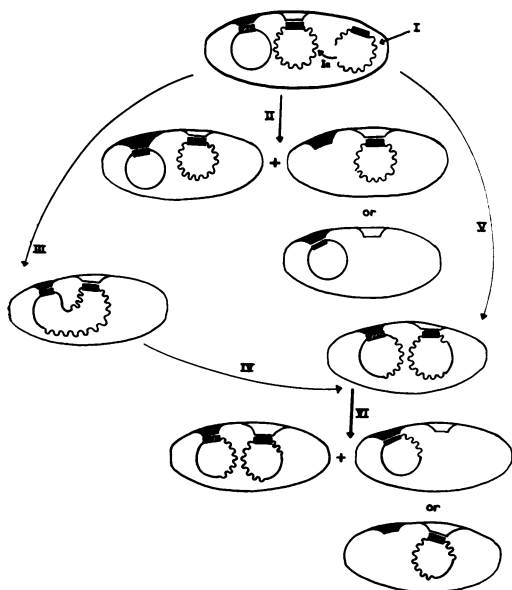


FIG. 14. Possible events in recombination between compatible plasmids. In this case the two elements are heterogenic at least for their attachment regions. Numbered stages are essentially the same as in Fig. 12, with the following exceptions: in II and IV, segregation ordinarily involves the appearance of a single homozygous plasmid without concomitant disruption of the heterodiploid; in III, it is assumed that the associated element occupies both attachment sites.

tionary significance in leading to the accretion of genetic substance as well as of clinical significance in possibly being involved in the assembly of complex R factor in the wild. The molecular basis of these recombination events is currently not well understood but may be approached through attempts to answer two questions: What is their basis in terms of base sequence homology? What are their topological consequences in terms of overall genetic structure of the products?

**Homology.** Between the extremes of isologous base sequences, which give maximal recombination, and unrelated ones, which give none, is a continuum of base sequence relatedness which gives rise to a corresponding continuum of recombination probability (viewed against a constant background of recombination-mediating enzymes). Two factors, the base sequence similarity of two regions and their length, must affect the probability of a crossover between them; long regions of low overall homology or very short ones of high homology could theoretically be equally likely to recombine. It seems certain that two heterogenic elements capable of recombining will differ markedly in homology along the lengths of their respective genomes and will thus have preferred or exclusive sites for crossing over.

**Topology.** Several topological possibilities exist for the assembly of more complex plasmids from simpler ones; their relative probabilities depend, among other things, upon homology relationships. Three of these possibilities are illustrated in Fig. 15a, b, and c, where the assembly of a plasmid carrying both markers A and B from two carrying A and B separately is used as an example. The parental plasmids are each shown carrying two other markers, R and F, which may or may not be homologous. In Fig. 15a, the two plasmids are homologous overall; defective A or B loci on one correspond to active ones on the other, and the recombination event is commutative. In Fig. 15b, the two are nonhomologous for A and B regions but homologous elsewhere; again, the recombination is commutative. In Fig. 15c, the two are homologous at only one region, F, and the recombination is associative, leading to a composite element with dual representation of genes F and R. Not knowing the history of any extant plasmid, one must bear in mind the possibility that it contains regions of internal homology that may have been acquired through events such as those in Fig. 15c. Such regions may lead to deletion of markers (Fig. 8) and to transposition, as shown in Fig. 15d.

Experimental evidence relevant to Fig. 15 has

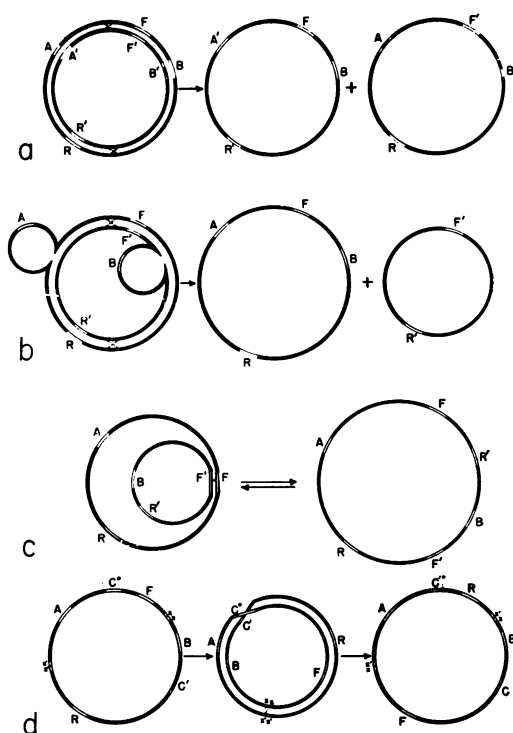


FIG. 15. Recombination possibilities involving heterogenic plasmids. Markers A, B, C, and F are somatic determinants including fertility. R is a determinant of replication. A-A', B-B', C-C', and F-F' represent allelic pairs. R and R' may or may not be allelic. Dark segments within loci mark defective genes. One-way arrows are used for reversible events whose products are stable; two-way arrows are used where the situation is inherently unstable. The "blisters" in part B indicate regions of one plasmid for which the other has no homologue. (a) Commutative recombination between fully homologous plasmids where one has a good A gene and a defective B allele, the other a defective A and a good B. The products have two good genes and two defective ones, respectively. (b) Commutative recombination between two partly homologous plasmids. One lacks an A region and the other lacks B. One product has both, the other is a "silent" sex factor. (c) Associative recombination between two plasmids that share only a small region, the F locus. The product has dual representation of F and R loci. (d) Intramolecular recombination involving a plasmid that has dual representation of a somatic marker, C; one copy is grossly defective (C') and the other has a point mutation (C\*). A second crossover involves a silent region of internal homology XX-X'X'. The product has a good C gene and a doubly defective one, as well as a transposition of F and R. A large variety of other products are possible, including deletions, depending on the location and number of regions such as XX and the number of crossovers that occur.

been obtained in R factors and in staphylococcal plasmids.

(i) Many workers studying R factors have tried diligently and unsuccessfully to isolate replication defectives (T. Wantanabe, *personal communication*; Y. Hirota, *personal communication*; S. Mitsuhashi, *personal communication*), a failure that might be accounted for by multiple representation of the replication genes. Deletions involving R factors often result in reduced but still detectable mating activity (145, 273, 277), an observation that is most easily explained on the basis of dual or multiple representation of the mating region accompanied by genetic drift among extra copies of duplicated loci. In contrast, Hashimoto and Hirota (102) and Hashimoto and Mitsuhashi (104) were able to obtain readily, with R(f)100, point mutations to chloramphenicol and tetracycline sensitivity. Since these alleles are recessive, the *tet* and *chl* genes must not have been multiply represented, at least in their active state.

(ii) In staphylococcus, evidence exists for multiple representation of the cadmium locus in one plasmid, PII<sub>147</sub>, but not in another, PI<sub>258</sub>. Cadmium-sensitive point mutants of PI<sub>258</sub> are quite stable and are usually fully sensitive. Cadmium-sensitive point mutants of PII<sub>147</sub> are never fully sensitive to the ion and are always unstable. Moreover, a large proportion of their cadmium-resistant reversions have suffered concomitant deletions for other plasmid markers (R. Novick, *unpublished data*). These reversions have been tentatively interpreted as representing internal recombination between two defective cadmium loci more or less as illustrated in Fig. 15d.

(iii) Nisioka et al. (188) and Falkow (Ciba Found. Symp., *in press*) recently found evidence that some R factors may be capable of alternating between existence as a single circular DNA molecule and two smaller ones. These observations may represent associative-dissociative recombination between independent replicons. In the example studied by Nisioka et al., there were apparently two sizes of small circles whose sum added up to that of the one large one—a finding that argues for a single strongly favored crossover site (see Fig. 15c and Table 5).

### Summary

Plasmid marker orders, where known, have been revealed mostly by the study of plasmid deletions. Because of sites of high deletion probability, map distances based on deletion frequencies are poorly representative of physical distances; this distortion can be corrected by physical measurements of DNA molecules. Recombination analy-

sis of isogenic (incompatible) plasmids is characterized by the apparent occurrence of paired crossovers within short regions of synapsis and is complicated by zygote asymmetry. The resident plasmid is on a footing distinctly different from that of the entering one, which results in a pronounced recipient bias among unselected markers, apparently owing to selective loss of one class of recombinants. With compatible and therefore heterogenic plasmids, the zygotes are stable and apparently symmetrical (both plasmids are on an equal footing); however, recombination analysis suffers from the drawback of heterogenicity affecting the most interesting region. Whether this will be a serious drawback is not yet clear. Characteristic of plasmid behavior is a variety of infrequent heritable variations that seem to be the result of crossovers between intra- or intermolecular regions of poor base sequence homology. These events, including accretion, loss, and rearrangement of markers, comprise a low-level, hereditary instability which may be characteristic of plasmids in general and of major significance in evolutionary flexibility.

## REPLICATION

### Mechanics of Replication

As the two DNA replication models currently being entertained, namely the Cairns model and the Rolling Circle model, are presumably applicable to plasmids they will be considered briefly in this review, partly in the hope that study of plasmids may help to clarify the overall picture of the replication process.

**Cairns model.** According to Cairns (28), the replicating chromosome is an integral structure that is firmly anchored, near the locus where replication is initiated, to a specific site in the cell. Initiation is by a signal coordinating the cell division cycle with replication (130, 148) and evidently involves the triggering of an energy-dependent unwinding mechanism, or "swivel" that requires a discontinuity in one strand for its operation. This swivel, an active mechanism distinct from DNA biosynthesis per se, transmits torque the entire length of the chromosome. The concept of the replicating chromosome as an integral structure is supported by the finding that a <sup>32</sup>P-induced scission anywhere in the *E. coli* chromosome immediately interrupts replication (29). The existence of an energy-requiring process in addition to the DNA biosynthetic reactions is supported by an observation of Cairns and Denhardt (30) that cyanide ion or carbon monoxide immediately stops chromosome replication but

permits synthesis of a complementary strand on a  $\phi$ X174 DNA template.

Demonstration of replicating forms of the *E. coli* (28) and *M. hominis* (19) chromosomes, liver mitochondrial DNA (144), coliphage lambda (197), and polyoma virus (117) has in all cases revealed structures topologically similar or identical to that originally identified by Cairns (28).

**Rolling circle model.** An alternative model has been formulated by Gilbert and Dressler (87) and is illustrated in Fig. 16 in terms of plasmid replication. As pictured (Fig. 16b), an entering plasmid, once cyclized, would be acted upon by a nucleotransferase complex that attacks one strand of the duplex at a sequence-specific site (*n*), and transfers the new 5' end to a specific receptor site on the cell membrane. This nucleotransferase would correspond to the proposed attachment protein. In the next stage (Fig. 16c), the Kornberg DNA polymerase would extend the free 3' end, using the intact strand as template and displacing the 5' end. When the free single strand was one genome long (Fig. 16d), the Kornberg enzyme would initiate a complementary copy starting at an initiation site (*i*) a short distance beyond the transferase site (*n*). As the enzyme evidently requires a short primer complementary to the template strand as well as a template (91), this step is the haziest, since the source of such a primer is unclear. Once the new strand has begun, the nucleotransferase would act again, leaving to be completed a linear duplex molecule with cohesive ends and attaching the new 5' end to a new membrane site (Fig. 16e). Cyclization of the linear copy by virtue of its cohesive ends plus ligase

action (85) to seal the gap in the unattached strand would complete the replication cycle (Fig. 16f). Since even the nucleotransferase complex could be provided by the host, this scheme lacks any essential function that is obligatorily plasmid-linked except for the two nucleotide sequences *i* and *n* in Fig. 16. Thus it would seem at best to be an oversimplification; several classes of plasmid mutants defective in functions apparently essential for replication have been isolated (48, 130, 193).

The rolling circle model predicts the occurrence of parental DNA strands longer than single length; the Cairns model without modification does not. Thus, experiments demonstrating redundant parental strands support the former. Such support has been provided by Gilbert and Dressler (87), who found redundant molecules among replicating  $\phi$ X174 genomes (87). Further, Matsubara (166) isolated linear F DNA, immediately after transfer of F to F<sup>-</sup> *E. coli* cells, and found it to be about twice as long as circular F; Goebel and Helinski (90) were able to increase the incidence of multiple-length rings of ColE1 DNA by inhibiting protein synthesis in the host cell (i.e., ostensibly inhibiting new initiation of replication but not propagation of chains already initiated). Occasional structures corresponding to Fig. 16d, consistent with rolling circle replication, have been seen in electron micrographs of replicating DNA (117, 144). The physical data consistent with redundant replication strengthen earlier observations such as that of Fulton (81) of redundant transfer of the Hfr chromosome.

Both models are supported by reliable observations, so that at present no choice between the two is strongly favored.

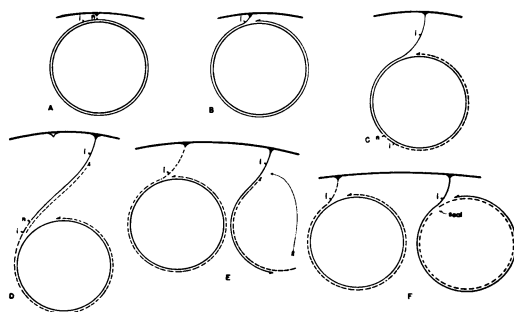


FIG. 16. Plasmid replication according to the "rolling circle" model. A specific site in one strand that is attacked by a special endonuclease that cleaves only one strand is indicated by *n*; the location of another special nucleotide sequence that serves as a recognition site for chain initiation by DNA polymerase is indicated by *i*; seal indicates a gap that is closed by polynucleotide ligase to complete the replication cycle. Free 3' ends are marked by arrows.

### Replication and Transfer

The first indication that conjugal transfer of DNA involves the breakage of a circular structure was the observation that the genetic map of *E. coli* is circular although the transferred structure is linear (134). Later, it was discovered the chromosome of *E. coli* is indeed physically circular (28), and it was proposed that the breakage of this structure, as a prerequisite of transfer, is a consequence of replication (129).

The role of DNA replication in conjugal transfer has been controversial for some time. This controversy has recently been reviewed by Falkow et al. (71), Scaife (230), Gross and Caro (92), and Cuzin et al. (47), and will not be discussed here. However, because of recent findings suggesting that single-stranded DNA is transferred during mating (41, 199, 224a, 269), I feel that a reevaluation is in order.

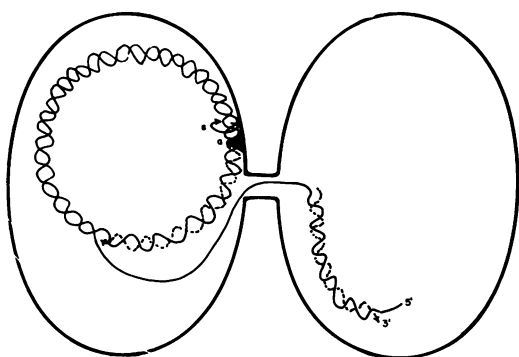


FIG. 17. Model for transfer replication. The donor chromosome is anchored to a maintenance site at *a* and is unwound by a swivel, *s*. Its free 5' end passes into the recipient while a new complementary strand is synthesized to replace it. Repair synthesis proceeds in the recipient.

Fig. 17 illustrates a possible mechanism of transfer replication based on Lark's modification of the Cairns model (148) and featuring the transfer of single-stranded DNA. The main departure of this modification from Cairn's model is that, at the initiation of replication, a 5' end is freed for attachment to a new maintenance site or, in this case, for insertion into the mating tube; in this respect the mechanism is similar to the rolling circle model. The concept of fixed attachment is retained and unwinding is still driven by a swivel.

In this scheme, true replication takes place in the donor (except that only one strand is copied there); the other strand is copied in the female by a process that resembles repair synthesis more than it does replication.

This conjectural model is intended to illustrate the value of assessing separately the role in transfer of various aspects of the replication process. Thus, DNA synthesis would occur in both donor and recipient as part of the conjugation process; for the physical transfer of DNA, however, polynucleotide synthesis might be required in neither. Repair synthesis in the recipient would presumably be involved in gene expression and in stabilization of donor DNA—either through recombination or through establishment as a plasmid. In the donor the requirement could be simply for unwinding and not for actual synthesis at all.

As an illustration, one would expect that either prevention of unwinding or inhibition of DNA synthesis upon a single-stranded template would stop DNA replication; yet the former, according to the model in Fig. 17, might not block conjugal transfer if conditions were arranged so that only the recipient would be affected. Conversely, the latter might not block conjugal trans-

fer if conditions were arranged so that only the donor would be affected.

The concept of separable functions is supported in principle by the behavior of two classes of thermosensitive *E. coli* mutants defective in DNA replication. Mutants of one class, tested at elevated temperature, are competent as recipients (47); the others are competent as donors but incompetent as recipients (21, 22).

Thus, differences between what the male does and what the female does during mating are amenable to differential blocking by mutations and perhaps by other means.

**Establishment following transfer.** Having entered as, or been converted to, linear duplex DNA, a plasmid evidently must next cyclize via cohesive ends or via recombination involving redundant double-stranded ends. Ikeda and Tomizawa (126) found that superhelical P1 prophage DNA molecules have a contour length of 32  $\mu$ m compared with a contour length of 37  $\mu$ m for the linear phage DNA, which is terminally redundant and does not have cohesive ends (263). Cyclization in this case can be imagined to occur by a specific intramolecular reciprocal crossover resulting in loss of the ends as fragments—although 5  $\mu$ m of terminal redundancy seems rather more than necessary. The plasmid must eventually attach to its maintenance site; studies with *E. coli* and *S. aureus* have revealed this step to be variable in time. Thus, after F-*gal* transfer to F<sup>-</sup> (96), about one-half of the zygotes give rise to pure Gal<sup>+</sup> daughter clones and one-half give rise to variegated Gal<sup>+</sup>/Gal<sup>-</sup>. During growth in broth after mating, the proportion of cells giving rise to variegated colonies diminishes as does the size of Gal<sup>-</sup> sectors. This finding requires the interpretation that, although F-*gal* establishes quite rapidly in a majority of the newly entered cells, full synchrony of plasmid and chromosome is often delayed and establishment may not involve a discrete event of short duration. Note that failure of some zygotes to multiply in broth prior to plating could lead to delayed disappearance of variegated clones but not to diminution in size of Gal<sup>-</sup> sectors. Staphylococcal plasmids are also slow to establish; after transduction, there is approximately a one-generation lag before the transductant population begins to increase (R. Novick, unpublished data). Individual transductant colonies isolated without selection seem to vary greatly with respect to the time a plasmid establishes. As with F-*gal*, such transductants contain plasmid-negative cells in widely varying proportions.

As discussed earlier in this paper, the presence of an incompatible plasmid poses a special obstacle to the establishment of a super-infecting one and may involve inhibition of replication (60;

R. Novick, *unpublished results*). Interest in this apparent replication arrest (term suggested by Werner Maas) centers around the stage at which plasmid establishment is held up and the specific sequence of events wherein the superinfecting element finally does stabilize. *F-lac*, on superinfecting an Hfr *recA* strain, becomes capable of phenotypic expression (in this case,  $\beta$ -galactosidase induction) shortly after entry (60). Evidently the entering plasmid is converted to a circular duplex form before it encounters the block, since linear DNA fragments [abortive transducing particles (109) and proximal segments of an Hfr chromosome (E. Dubnau and W. Maas, *Mol. Gen. Genet.*, *in press*)] are unable to function (are destroyed?) in *recA* hosts. Thus, *F-lac* is evidently arrested just prior to maintenance-site attachment.

### Control of Replication

**Dual control for vegetative plasmids.** A discussion of the control of vegetative phage replication is beyond the scope of this paper. (See review by Echols and Joyner, 64.) However, as there are certain plasmids that exist either in a quiescent autonomous state or replicate vegetatively, a short comment on a possible basis for control of such alternating replication is relevant.

The best-known examples are nontransmissible Col factors, such as ColE1, and phages, such as P1, whose prophage replicates autonomously. Presumably such plasmids operate a set of vegetative functions, including those for DNA synthesis, that are repressed in the quiescent state. Nevertheless, they are required to replicate autonomously and in synchrony with the host division cycle while under this repression. It seems a priori unlikely that a set of lethal vegetative functions could be tuned finely enough under repressive control to allow precisely one round of replication per cell division cycle. Alternatively, it would seem more likely that there is a separate control system for quiescent replication that is basically similar to control systems for wholly quiescent plasmids and is unaffected by repression of vegetative functions. This system would involve maintenance site attachment, initiation, etc., and would be superseded by the vegetative replication system upon induction.

This dual control model predicts for phages such as P1 that it should be possible to isolate mutants specifically affecting the quiescent replication cycle. Such mutants should give rise to abortive lysogens. Conversely, mutations affecting vegetative DNA replication should not affect ability to exist as a prophage. Similar predictions can be made for plasmids such as ColE1 but will be more difficult to verify experimentally.

A somewhat artificial example of alternating replication control has been observed in staphylococcus (194) with an Hft element. This element, P11de, originated as a recombinant between two heterogenic replicons: a generalized transducing phage, P11, and a plasmid, PI<sub>258</sub>. Though P11de includes a segment of the P11 genome as well as the plasmid *ero* and *mcr* loci (see Fig. 9), it is cryptic for phage functions. When vegetative P11 is not present in the cell, P11de behaves like a plasmid in that its quiescent replication cycle is under the control of its plasmid *mcr* region. When a P11de strain is superinfected by active P11, the superinfecting phage induces the defective P11de to replicate extensively; the resulting lysate is Hft for erythromycin resistance. Similarly, P1 prophage is apparently induced to replicate by superinfecting virulent P1 (C. Shemin, Ph.D. thesis, Massachusetts Institute of Technology, 1967).

It has been assumed that ColIb-P9 and other colicinogenic sex factors alternate between quiescent and vegetative replication. This assumption is based on the effects of UV on colicin (1, 183) and DNA (2) synthesis in ColI<sup>+</sup> strains. UV induction of colicin I synthesis is at best controversial (183, 206), apparently occurring in some strains but not in others (1). In the cases where it has been seen, the possibility of release of colicin contingent on the induction of an unsuspected phage has not been ruled out. Mitomycin C does not induce colicin I or V synthesis in *E. coli* under conditions where ColE1 is very effectively induced (107). The evidence for lethal synthesis of colicin I and for UV stimulation of fertility (183) is shaky, and the report of Amati (2) that ColI replicates vegetatively after UV irradiation of its host can be discounted because ColI carries a marker that protects its host against UV irradiation (122). Thus there is no compelling reason to consider colicinogenic sex factors as anything more than transmissible plasmids carrying determinants of colicinogeny and colicin resistance.

**Quiescent plasmids—summary.** Our current picture of the genetic control of plasmid replication has a substantial theoretical foundation in the replicon and maintenance site hypotheses of Jacob et al. (130). However, although certain key features of these hypotheses are supported by experimental observations, the overall theory is by no means proven, and detailed information is scarce indeed. Thus various lines of evidence support the concept of specific maintenance sites, and the isolation of host mutants defective in maintenance of plasmids belonging to a single incompatibility set proves that the host has a specific role; likewise, the demonstration of plasmid-linked

functions essential for autonomy (130, 193) proves that plasmids are replicons. But the number and nature of essential plasmid functions and the nature of the host maintenance system are unknown—it is not clear whether any defective mutant plasmid is blocked specifically in autonomous replication, in distribution, or in some other unsuspected essential function; nor is it clear whether the host mutants have defective maintenance sites. Very indirect evidence suggests that one staphylococcal *seg*<sup>-</sup> plasmid is defective in attachment to its maintenance site, but much needs to be done before a final assessment can be made. It can also be suggested on the basis of indirect evidence that one *F*<sub>t</sub> - *lac* mutant is not defective in maintaining its attachment (49) but available evidence does not indicate whether the *F*<sub>t</sub> - *lac* can form its attachment at high temperature. Regarding the number of plasmid-linked essential functions, Cuzin and Jacob (48) reported, among *F*<sub>t</sub> - *lac* mutants and fertility-defective Hfr strains at least three separate complementation groups involved in autonomous replication of *F*-*lac*. However, these results are in doubt, because complementation was tested in *rec*<sup>+</sup> Hfr strains superinfected with *F*-*lac* by making use of the rare clones where the two somehow coexist. In view of the inability of Dubnau and Maas (60) to isolate stable Hfr/*F*-*lac* by superinfection of an Hfr *recA* strain, this "complementation" may reflect stabilization of the *F*<sub>t</sub>-*lac* through integration.

Finally, it might be imagined that, if replication is required for genetic transfer, sex factor mutants defective in autonomous replication would also be infertile; thus, the finding that *F*<sub>t</sub>-*lac* mutants are able to transfer normally at elevated temperature (130) is paradoxical.

### CONCLUSION

This review has tried to sketch broadly a coherent overview of the bacterial plasmids as a well-defined biological family, wherein similarities far outweigh differences. The emphasis has been on the nature and the precise role of a set of functions common to all plasmids, namely those involved in the achievement and maintenance of the autonomous state. Thus the universal occurrence of incompatibility between isogenic plasmids probably reflects a fundamental similarity among stable bacterial replicons with respect to the mechanism of replication and the segregation of replicas. This mechanism may well be a prototype of the corresponding mechanism in all higher forms. Whether this general similarity among plasmids will hold true in detail awaits explorations of the genetic control of plasmid replication, just now beginning. Similarities among plasmids

go beyond the essential functions. The widespread carriage of genes that protect host organisms against an inimical environment must surely have a profound evolutionary significance, a significance that may reflect a truly symbiotic relationship rather than a parasitic one between plasmid and host.

Plasmids are also similar in their ability to exchange genes with one another and with other genetic elements in the cells that they inhabit. This ability reflects in part a widespread low-grade genetic homology among plasmids of a particular organism, but it also reflects another similarity, namely topographical organization of plasmid molecules into genetically essential and nonessential regions. Such organization lowers the risk inherent in crossovers due to low homology since, were essential genes dispersed, such crossovers might involve them in excision loops or in exchanges for nonessential ones (see Fig. 15).

Perhaps akin to widespread recombination is the evolution and proliferation of conjugal transmissibility among plasmids. This biologically unique situation, a transmissible agent determining sexual differentiation, attests to the importance of plasmids in the life of the bacterium and its geneticist.

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